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(54) Title: GERMLINE-COMPETENT AVIAN CELLS

(57) Abstract

Methods for identifying germline-competent avian cells, such as cultured chicken blastodermal cells, and methods for generating transgenic avians are disclosed. In addition, methods of screening for cell culture conditions or factors which promote the maintenance of germline-competent avian cells or other germline-competent animal cells in culture are also disclosed. Used in the methods of the invention are expression constructs comprising (i) a marker gene; and (ii) control sequences sufficient for effecting expression of the marker gene in germline-competent cells, wherein at least one of the control sequences comprises a control sequence of a gene which is substantially expressed only in cells which are germline-competent. The expression constructs contain a *Oct4* proximal promoter.

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GERMLINE-COMPETENT AVIAN CELLS

BACKGROUND OF THE INVENTION

a) Field of the Invention

The present invention relates generally to the field of animal transgenesis. More specifically, the invention relates to vectors and methods for identifying germline-competent animal cells and, in particular, germline-competent avian embryonic cells, which are useful in the generation of transgenic animals. The invention also relates to methods of screening cell culture conditions for those which promote the maintenance of germline competence in cells over extended periods of time.

b) Description of Related Art

The production of transgenic animals by introduction of exogenous genes into their germline has been achieved for a number of different animals including mice, cattle, rabbits, pigs, sheep, and fish. The introduction of exogenous genes into an animal's genome allows for the modification of the phenotypic characteristics of the animal. For example, the introduction of an appropriate transgene can potentially increase the disease resistance, growth rate, muscle mass or the like of an animal.

The production of transgenic birds is likewise highly desirable. Altering the avian genome can lead to the generation of desirable phenotypes. Furthermore, appropriate modification of the avian genome can lead to the production of exogenous protein within the oviduct followed by deposition of the exogenous proteins in the eggs of the bird. Use of the chicken as a bioreactor for the production of therapeutic proteins has significant advantages over the common methods of isolating proteins from natural sources and producing recombinant proteins in bacterial or mammalian cells.

Many attempts to introduce an exogenous expression construct into birds have involved the injection of retroviruses carrying non-viral transgenes into a freshly laid egg, just below the blastoderm (for examples, see Bosselman *et al.*, *Science*, 1989, 243: 533-535; Salter *et al.*, *Virology*, 1987, 157:236-240; Hughes *et al.*, US Patent No. 4,997,763). Although, some success has been achieved using these procedures, complications can ensue. In some cases, efficient transduction of germline cells or expression of the inserted retroviral transgene has been problematic. In other cases, when the retroviral vector used is replication-competent, the genetically-modified chickens are viremic. Also, the size of the transgene in the retroviral vector is greatly limited.

In a different approach towards genetically altering birds, chimeric chickens have been generated by the injection of chicken blastodermal cells from one embryo into a recipient embryo, usually a stage X embryo. The donor blastodermal cells used in these experiments have been shown to be able to contribute to both somatic tissues (Watanabe *et al.*, *Development*, 1992, 114:331-338; Fraser *et al.*, *Int. J. Devel. Biol.*, 1993, 37:381-385) and the germline (Thoraval *et al.*, *Poultry Sci.*, 1994, 73:1897-1905; Carsience *et al.*, *Development*, 1993, 117:669-675; Petitte *et al.*, *Development*, 1990, 108:185-189) of the resulting chimeras.

Although transgenic chickens can in theory be readily generated by the genetic manipulation of the donor embryonic cells prior to injection to the recipient blastoderm, the situation is greatly complicated by the fact that many sophisticated genetic manipulations require that the cell be maintained in culture over a period of time while the cells are screened for successful transfection, integration, or orientation of the transgene vector. In such experiments, it would be highly desirable to be able to culture the explant blastodermal cells for a sufficient amount of time to allow for the multiplication of embryonic stem cell progenitors without differentiation. However, it is very difficult to culture chicken embryonic or blastodermal cells for any period of time over approximately four

days due to the tendency of chicken blastodermal cells to lose their ability to contribute to germline tissues of recipient embryos when cultured *in vitro*.

Examples of the difficulties of maintaining germline competence of chicken blastodermal cells in culture are detailed in Etches *et al.*, *Mol Reprod Dev,* 1996, 45:291-8. In Etches *et al.*, 1996, the frequency of contribution of cultured chicken blastodermal cells to germline and somatic chimeras upon injection to recipient embryos was compared to that of fresh chicken blastodermal cells. The chicken blastodermal cells were cultured under a variety of different conditions for 48 hours. Subjecting the chicken blastodermal cells to culturing for only 48 hours under any of the conditions tested resulted in a drop in germline contribution of over 60%.

Because of the problem of maintaining totipotent avian embryonic cells in culture, various attempts have been made by researchers to develop an effective method of determining the germline competence of the cells before addition to a recipient embryo. Several assays which attempt to identify totipotent chicken blastodermal cells have been reported (Karagenc *et al.*, *Dev. Genet.*, 1996, 19:290-301; Pain *et al.*, *Development,* 1996, 122:2339-2348; Urven *et al.*, *Development,* 1988, 103:299-304). These assays involve the detection of specific proteins thought to be characteristic of totipotent chicken blastodermal cells. Monoclonal antibodies specific to stage-specific embryonic antigen-1 (SSEA-1) or embryonal carcinoma Nulli SCC1 (EMA-1) are used in some of the assays (Karagenc *et al.*, 1996; Urven *et al.,* 1988). In Pain *et al.,* 1996, the targeted protein is alkaline phosphatase. Such attempts are demonstrative of the need to sort germline-competent avian cells from those avian cells which have differentiated.

Similarly, due to the problems of maintaining avian embryonic cells in culture, attempts have also been made to alter the culture conditions to promote the stability of chicken embryonic cells in culture. For instance, U.S. Patent No. 5,656, 479, Petitte *et al.*, teaches a procedure of growing avian stem cells on a mouse fibroblast feeder layer in the presence of a medium containing leukemia

inhibitory factor (LIF) to generate a sustained avian stem cell culture. The success of such a procedure, however, is limited. For example, the Petitte *et al.* procedure was one of those tested in Etches *et al.*, *Mol. Reprod. Dev.*, 1996, 45:291-8, as described above.

In contrast to the case with chicken blastodermal cells, there has been considerable success in maintaining certain strains of murine embryonic stem cells *in vivo*. Extensive experiments with murine embryonic cells and murine stem cell culture have led to the identification of a transcription factor which is specifically expressed in the totipotent cells of the early mouse embryo and in undifferentiated mouse embryonic stem (ES), embryonal carcinoma (EC) cells, and embryonic germ (EG) cells (Minucci *et al.*, *Embo J.*, 1996, 15:888-99; Okamoto *et al.*, *Cell*, 1990, 60:461-72; Okazawa *et al.*, *Embo J.*, 1991, 10:2997-3005; Yeom *et al.*, *Mech. Dev.*, 1991, 35:171-9; Shimazaki *et al.*, *Embo J.*, 1993, 12:4489-98; Brehm *et al.*, 1998). This transcription factor is the mouse octamer-binding transcription factor 3/4, referred to as Oct4 herein.

Oct4 is a member of the POU family of transcription factors. POU factors contain a bipartite DNA-binding domain. The N-terminal domain consists of approximately 75 amino acids and the C-terminal domain consists of approximately 60 amino acid residues. The two domains are connected by a linker that varies in both sequence and length among members. The *Oct4* gene is split into five exons and encodes a transcript of about 1.5 kb. Expression of *Oct4* in the embryo is activated before the 8-cell stage and has been detected in the extracts of complete mouse embryos as late as 9.5 days post coitum.

In Yeom *et al.*, *Development*, 1996, 122:881-94 two separate regulatory elements located upstream of the *Oct4* gene are identified (see Fig. 1A). One regulatory region contains the proximal enhancer (PE) which is located about 1.2 kb upstream of the transcription initiation site and is necessary for epiblast expression and retinoic acid-mediated downregulation of *Oct4*. The second regulatory region of *Oct4* is the distal enhancer (DE) which is located

approximately 2 kb upstream of the transcription initiation site. This element is active only in germline-competent cells such as those in the preimplantation embryo. Hence, the distal enhancer is responsible for *Oct4* expression in ES cells and EG cells in mice.

A basal promoter is also present upstream of the transcription initiation site. A GC box to which members of the Sp1 family can bind is located approximately 30 base pairs upstream. A hormone response element (HRE) partially overlaps this GC box.

Because its expression has been shown to be limited to undifferentiated stem and germ cells, the mouse *Oct4* promoter has been combined with heterologous genes in expression vectors and used to identify germline-competent embryonic stem (ES) cells in mammals. For instance, McWhir *et al.*, 1996, *Nat. Genetics* 14:223-6 reports the use of an expression vector combining the *Oct4* promoter with a neomycin resistance gene in mouse ES cells to select for those which are germline-competent. Similarly, WO 94/24274, Smith *et al.*, describes a method to select for pluripotent embryonic stem cells by introducing a vector with the *Oct4* promoter linked to a selectable gene, such as the neomycin resistance gene, into embryo cells and selecting for cells that are resistant to drug treatment and thus are pluripotent. Yeom *et al.*, *Development*, 1996, 122:881-94, combines the *Oct4* promoter region with the bacterial *lacZ* gene to establish expression patterns in mouse embryos.

To date, an avian analog of the murine Oct4 protein has not been found. Interspecies hybridization analysis using a probe corresponding to part of the murine *Oct4* N-terminus sequence indicated that at least this portion of the *Oct4* sequence is only conserved in mammals (Yeom *et al.*, *Mech. Dev.*, 1991, 35:171-9). In these experiments, no Oct4 related sequences were found in chicken and other vertebrates such as frog and fish.

Thus, there exists a need for a reliable method of identifying avian cells which possess the ability to contribute to germline tissue. There also exists a need

to be able to screen cell culture conditions and factors for those which will effectively promote continued germline competence of cells in culture over extended periods of time.

SUMMARY OF THE INVENTION

The present invention provides methods of identifying avian cells which possess the ability to give rise to germline tissue. The invention also provides methods of screening factors in cell culture for those which promote the maintenance of germline competence in cells in culture over time. Vectors useful in these methods and cells and cell cultures generated by these methods are also provided.

In one embodiment, the present invention provides for a method of identifying avian cells which can give rise to germline tissue. This method comprises first introducing an expression construct into at least one avian cell. The introduced expression construct comprises a marker gene operably linked to control sequences sufficient for effecting expression of the marker gene in germline-competent cells, wherein the control sequences comprise at least one control sequence from a gene which is substantially expressed only in cells which are germline-competent. The control sequence from a gene which is substantially expressed only in cells which are germline-competent may comprise an element like an enhancer or promoter, or a combination thereof. In a second step of the method, the cells are assayed for expression of the marker gene in the cells. Expression of the marker gene in a cell indicates that that cell can give rise to germline tissue.

In one embodiment of the invention, the introduced expression construct then comprises an *Oct4* proximal promoter and a marker gene, which are operably linked. The second step of the method involves assaying for expression of the

marker gene in the cells which contain the expression construct. The expression of the marker gene in a particular cell indicates that that particular cell can contribute to germline tissues, such as the germline tissue of a recipient embryo, for instance.

The introduced expression construct may optionally also comprise the distal enhancer or the proximal enhancer of the *Oct4* gene or both or even the complete upstream control region. In one embodiment, the *Oct4* enhancer is derived from the proximal or distal enhancer of the mouse *Oct4* gene. In a preferred embodiment, the promoter on the expression construct is derived from the mouse *Oct4* gene.

In a preferred embodiment, the avian cells are avian embryonic cells such as chicken blastodermal cells. Optionally, the avian embryonic cells are cultured cells.

The present invention also provides for a method of producing a germline-modified transgenic avian animal. This method comprises introducing into avian embryonic cells a transgene of interest either before, after, or at the same time as introducing into the cells an expression construct that contains a marker gene operably linked to control sequences sufficient for effecting expression of the marker gene in cells which are germline-competent, wherein the control sequences comprise at least one control element from a gene which is substantially expressed only in cells which are germline-competent. Those avian embryonic cells which comprise the transgene of interest and also express the marker gene are identified and transferred to an avian embryo, where at least some of the transferred cells contribute to the germline tissue of the avian embryo. As a final step, the recipient avian embryo is allowed to develop to hatch. The transgene is present in the germline tissue of the resulting avian animal.

Preferably, the control sequences comprise a proximal promoter from the *Oct4* gene.

In a preferred embodiment, the transgene becomes integrated into the genome of the cells which make up the germline tissue of the avian embryo.

In a further preferred embodiment, the avian embryonic cells are cultured or uncultured chicken blastodermal cells.

A method of creating a germline-modified chimeric avian animal is also contemplated by the present invention. The method involves first introducing into avian embryonic cells an expression construct containing a marker gene operably linked to control sequences sufficient for effecting expression of the marker gene in germline-competent cells, wherein the control sequences comprise at least one control element from a gene that is substantially expressed only in cells which are germline-competent. For instance, the marker gene may be operably linked to an *Oct4* proximal promoter. Next, those cells which express the marker gene are identified. The identified cells are then transferred to a recipient avian embryo, where they contribute to the germline tissue of the recipient avian embryo. The avian embryo is then allowed to develop to hatch.

In an alternative embodiment, the invention provides for a method of screening various cell culture conditions for those which allow for the continued maintenance of the germline competence of cells in culture over time. This method involves first subjecting a population of germline-competent cells to a set of selected cell culture conditions for a selected period of time. After the selected period of time has elapsed, an expression construct is introduced into the cultured cells. This introduced expression construct comprises a marker gene operably linked to control sequences sufficient for effecting expression of the marker gene in germline-competent cells, wherein the control sequences comprise at least one control sequence from a gene which is substantially expressed only in cells which are germline-competent. Preferably, an *Oct4* promoter is used as a control sequence in the expression construct. Next, the population of cells is assayed for expression of the marker gene. The expression of the marker gene indicates that the cells in the population are able to contribute to the germline and that the

selected culture conditions allow for the culture of germline-competent cells over the selected period of time.

In one embodiment, the cells which are screened may be either stem cells or germ cells. The cells may be avian cells such as chicken blastodermal cells. Alternatively, the cells may be mammalian or otherwise non-avian.

In a preferred embodiment of the cell culture screening method, the steps of the method are applied to a plurality of cell populations in parallel, each of the cell populations being subjected to a different selected culture condition.

The present invention also provides a method of selecting for germline-competent avian cells. This method comprises first introducing into a plurality of avian cells an expression construct comprising an operably linked *Oct4* proximal promoter and selectable marker gene. Next, the plurality of cells are subjected to conditions in which the expression of the selectable marker gene in the cell is required for survival of the cell.

The present invention also provides an avian cell which comprises an expression construct containing a marker gene operably linked to control sequences sufficient for effecting expression of the marker gene in germline-competent cells, wherein the control sequences comprise at least one control sequence from a gene which is substantially expressed only in cells which are germline-competent. For instance, the avian cell may contain an expression construct which comprises an operably linked combination of an *Oct4* proximal promoter and a marker gene. Avian cell cultures which comprise the avian cells of the invention are further contemplated. A germline-competent avian cell characterized by expression of a marker gene which indicates germline competence is also provided.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the map and sequence of portions of the mouse *Oct4* gene including the upstream control region. In Figure 1A, critical control sequences are indicated by the striped boxes. The *Oct4* open reading frame (ORF) is indicated by the stippled arrow. The first number in parentheses is the nucleotide position relative to the beginning of the map. The second number in parentheses is the nucleotide position relative to the first transcription site. In Figure 1B, the combined DNA sequence of the *Oct4* enhancer and promoter used in the expression vector pOCT4-GFP-PE (Figure 2A) is shown (SEQ ID NO:1). This sequence includes both the *Oct4* proximal enhancer and the *Oct4* proximal promoter.

Figure 2 shows the map of the *Oct4*/marker expression vectors, pOct4-GFP-PE (Figure 2A) and pOct4-GFP-DE (Figure 2B). The proximal enhancer, distal enhancer, and promoter are indicated by the striped boxes. The enhanced green fluorescent protein (EGFP) open reading frame is indicated by the stippled arrow.

Figure 3 shows the expression of enhanced green fluorescent protein (EGFP) under control of the *Oct4* enhancer and the *Oct4* proximal promoter in chicken blastodermal cells and subsequent downregulation of the *Oct4* promoter with longer periods of culture. Figure 3A shows chicken blastodermal cells which have been transfected with pOct4-EGFP-PE, and cultured for 24 hours. Figure 3B shows the fluorescent image of the field shown in Figure 3A. Fluorescing cells are expressing GFP. Figure 3C shows chicken blastodermal cells which have been cultured for 6 days, transfected with pOct4-EGFP-PE and then cultured for an additional 24 hours. The fluorescent image of the field shown in Figure 3C is shown in Figure 3D.

Figure 4 demonstrates the correlation between the expression of the *Oct4*/marker expression construct in cultured chicken blastodermal cells and

ability of those chicken blastodermal cells to contribute to germ tissues in chimeras.

Figure 5 shows the map of the *Oct-4* vector, pOct4-EGFP-PP. The proximal promoter is indicated by the striped box. The EGFP open reading frame is indicated by the stippled arrow.

Figure 6 provides the nucleotide sequence of the mouse *Oct-4* proximal promoter, used in the pOct4-EGFP-PP vector.

DETAILED DESCRIPTION OF THE INVENTION

a) Definitions and General Parameters

The following definitions are set forth to illustrate and define the meaning and scope of the various terms used to describe the invention herein.

A "germline-competent" cell is a cell which can contribute to germline tissue. A germline-competent cell may, but need not necessarily be, a totipotent cell. Alternatively, a germline-competent cell can be a pluripotent cell.

A "totipotent" cell, as used herein, is a cell capable of giving rise to all types of differentiated cells found in the particular organism from which the cell originated. A totipotent cell is also a cell capable of contributing to germline tissue.

A "pluripotent" cell is a cell capable of differentiating into more than one different final differentiated types. A pluripotent cell may or may not be capable of contributing to germline tissue.

"Germline tissue", as used herein, refers to cells of the reproductive organs from which sperm or oocytes are formed.

A "marker gene" is a gene which encodes a protein that allows for identification and isolation of correctly transfected cells. Suitable marker sequences include, but are not limited to those encoding green, yellow, and blue fluorescent protein (the *GFP, YFP*, and *BFP* genes, respectively). Other suitable markers include genes encoding thymidine kinase (tk), dihydrofolate reductase (DHFR), and aminoglycoside phosphotransferase (APH). The latter imparts resistance to the aminoglycoside antibiotics, such as kanamycin, neomycin, and geneticin. Use of a neomycin resistance gene as a marker is particularly suitable. Other marker genes include those encoding chloramphenicol acetyltransferase (CAT), β-lactamase, and β-galactosidase (β-gal). A "reporter gene" is a marker gene that "reports" its activity in a cell by the presence of the protein that it encodes.

The term "*Oct4* enhancer" refers to any enhancer derived from the control sequences of the *Oct4* gene. The term includes, but is not limited to, one of the two enhancer elements which have been identified upstream of the coding sequence of the *Oct4* gene. The two identified enhancer elements are referred to as the "distal enhancer" or "*Oct4* distal enhancer" and the "proximal enhancer" or "*Oct4* proximal enhancer". The proximal enhancer refers to the enhancer which is positioned closest to the promoter and transcription start site of the *Oct4* gene. The distal enhancer is the enhancer which is positioned further upstream. In the mouse genome, the distal enhancer comprises the nucleotide region stretching from the *Bam*HI site at -4330 to the *Bam*HI site at -1220 (where the beginning of the first transcription site of the *Oct4* gene is denoted +1; see Okazawa *et al.*, 1991, and Figure 1A). In the mouse genome, the proximal enhancer comprises the nucleotide region from the *Bam*HI site at -1220 to the *Bst*EII site at -224. Although both enhancer elements are preferably derived from the mouse *Oct4* gene, they may also be derived from *Oct4* genes of other mammals or other vertebrates.

The term "*Oct4* proximal promoter" refers herein to the promoter region which is proximal to the *Oct4* gene. The sequence is preferably, but not necessarily, derived from the mouse *Oct4* gene shown in Figure 6.

"*Oct4* enhancer/promoter" refers to an operably linked combination of an *Oct4* enhancer and promoter.

A gene which is "substantially expressed only in cells which are germline-competent" is a gene which shows at least an approximately 5-fold higher level of expression (as evidenced by protein levels) in cells which are germline-competent than in cells which are not germline-competent under a given set of conditions. Preferably, the gene shows at least an approximately 10-fold higher level of expression in cells which are germline-competent than in cells which are not germline-competent.

"Operably or operatively linked" refers to the configuration of the coding and/or control sequences so as to perform the desired function. Thus, control sequences operably linked to a coding sequence are capable of effecting the expression of the coding sequence. A coding sequence is operably linked to or under the control of transcriptional regulatory regions in a cell when RNA polymerase will bind the control sequence and transcribe the coding sequence into mRNA that can be translated into the encoded protein. The control sequences need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between a control sequence and the coding sequence and the promoter sequence can still be considered "operably linked" to the coding sequence. Furthermore, when a control sequence (such as an enhancer) is said to be operably linked to another control sequence (such as a promoter), the sequences are capable of working together to regulate expression of the coding sequence.

The term "control sequences" refers herein to nucleic acid sequences which control transcription of a given gene. For instance, control sequences include those sequences required to initiate or terminate gene transcription plus those sequences which (positively or negatively) regulate the rate at which transcription initiation occurs. Examples of control sequences in eucaryotic cells include, but are not limited to, promoters, enhancers, and repressor binding sites.

"Vector" means a polynucleotide comprised of single strand, double strand, circular, or supercoiled DNA or RNA. A typical vector may be comprised of the following elements operatively linked at appropriate distances for allowing functional gene expression: replication origin, promoter, enhancer, 5' mRNA leader sequence, ribosomal binding site, nucleic acid cassette, termination and polyadenylation sites, and selectable marker sequences. One or more of these elements may be omitted in specific applications. The nucleic acid cassette can include a restriction site for insertion of the nucleic acid sequence to be expressed.

In a functional vector the nucleic acid cassette contains the nucleic acid sequence to be expressed including translation initiation and termination sites. An intron optionally may be included in the construct, preferably ≥ 100 bp 5' to the coding sequence.

A "coding sequence" refers to a polynucleotide or nucleic acid sequence which can be transcribed and translated (in the case of DNA) or translated (in the case of mRNA) into a polypeptide *in vitro* or *in vivo* when placed under the control of appropriate control sequences. The boundaries of the coding sequence are determined by a translation start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A transcription termination sequence will usually be located 3' to the coding sequence. A coding sequence may be flanked on the 5' and/or 3' ends by untranslated regions.

An "expression construct" or "expression vector" is a vector which is constructed so that the particular coding sequence is located in the vector with the appropriate control sequences including regulatory sequences, the positioning and orientation of the coding sequence with respect to the control sequences being such that the coding sequence is transcribed under the "control" of the control or regulatory sequences. Modification of the sequences encoding the particular protein of interest may be desirable to achieve this end. For example, in some cases it may be necessary to modify the sequence so that it may be attached to the control sequences with the appropriate orientation; or to maintain the reading frame. The control sequences and other regulatory sequences may be ligated to the coding sequence prior to insertion into a vector. Alternatively, the coding sequence can be cloned directly into an expression vector which already contains the control sequences and an appropriate restriction site which is in reading frame with and under regulatory control of the control sequences.

An "*Oct4*/marker expression construct" or "*Oct4*/marker expression vector" is defined herein as an expression construct comprising an *Oct4* proximal

promoter and a marker gene, and optionally an *Oct4* enhancer wherein the *Oct4* enhancer, the promoter, and the marker gene are all operably linked.

b) Expression constructs of the invention.

The primary expression construct used in all of the methods of the present invention comprises (i) a marker gene and (ii) control sequences operably linked to the marker gene and sufficient for effecting expression of the marker gene in germline-competent cells, wherein the control sequences comprise at least one control sequence from a gene which is substantially expressed only in cells which are germline-competent. Typically, the control sequences sufficient for effecting expression of the marker gene in germline-competent cells may comprise multiple control sequences, such as a combination of a promoter and an enhancer. Alternatively, in other embodiments, a single control sequence, such as a promoter, may be sufficient for effecting expression of the marker gene in germline-competent cells. The control sequence which is from a gene that is substantially expressed only in cells which are germline-competent may optionally comprise an enhancer, a promoter, or both. Alternatively, the control sequences of the expression construct may comprise a negative regulatory element which is active only when a cell is differentiated.

In a preferred embodiment, the expression construct is an *Oct4*/marker expression construct. This construct comprises an *Oct4* proximal promoter, and a marker gene, where the *Oct4* proximal promoter, and the marker gene are operably linked.

In one embodiment, the vector further comprises an *Oct4* enhancer derived from the upstream region of the mouse *Oct4* gene (Okazawa *et al.*, *Embo J.*, 1991; 10:2997-3005; Yeom *et al.*, *Mech. Dev.*, 1991, 35:171-9). The *Oct4* enhancer may optionally be either the distal or the proximal enhancer of the mouse *Oct4* gene. We have established that the murine *Oct4* prosimal promoter either by itself, or with an enhancer, is active in avian embryonic cells, such as chicken

blastodermal cells isolated from chicken embryos (see Examples 2 and 3, below, and Figures 3 and 4). It has also now been determined that in cultured chicken blastodermal cells that have lost the ability to contribute to germline tissues of recipient embryos, the mouse *Oct4* promoter is inactive (see Example 3, below, and Figure 4).

The ability to use the control sequences of the murine *Oct4* gene to indicate germline competence in avian cells was unexpected because of the general lack of similarity between birds and mammals and because no functional chicken homologue or other non-mammalian homologue of the mouse *Oct4* has yet been identified, although the gene has been identified in a number of mammals such as rats, cats, minks, and humans (Yeom et al., 1991; Brehm *et al.*, *APMIS*, 1998, 106:114-126. *Oct4* has been described in the literature as a "marker of the mammalian germline" (Brehm *et al.*, 1998) and, to our knowledge, no evidence has previously been presented that the gene either does or could retain such a function in a non-mammalian species.

In an alternative embodiment, now that it has been established that *Oct4* control sequences from non-avian animals retain their functional specificity in avian animals, *Oct4* promoter sequences which have been identified from animals other than mice may instead be used in the *Oct4*/marker expression construct. For instance, *Oct4* gene sequences have been identified for rat, cat, mink, and human. A promoter from any of these sequences or from avian animals (when identified) may optionally be substituted into the *Oct4*/marker expression construct in place of a mouse *Oct4* promoter.

In a preferred embodiment of the invention, the promoter on the *Oct4*/marker expression construct is derived from the basal promoter region proximal to the transcription start site of the *Oct4* gene (the "*Oct4* proximal promoter"). In a particularly preferred embodiment, the *Oct4* proximal promoter is from the mouse genome.

In one embodiment, the control sequences of the *Oct4*/marker expression construct comprise approximately 1.9 kb of the region upstream of the transcription start site of the mouse *Oct4* gene (Okazawa *et al.*, *Embo. J.*, 1991, 10:2997-3005). In another embodiment of the invention, the sequences of the *Oct4* enhancer and the promoter of the expression construct are derived from the nucleotides ranging from approximately -1879 to approximately +29 of the mouse *Oct4* gene sequence and its upstream region (Okazawa *et al.*, 1991). This region extends from the *Hind*III site at -1877 to just downstream of the second transcription start site (Figure 1A). The preferred sequence for the *Oct4* enhancer/promoter combination is shown in Figure 1B (SEQ ID NO:1). This sequence comprises both the *Oct4* proximal enhancer and the *Oct4* proximal promoter. Various other functional derivatives of the 1.9 kb mouse *Oct4* promoter region may also be employed in the *Oct4*/marker expression construct.

In Figure 2A, an expression construct is shown which contains both the *Oct4* proximal promoter and the *Oct4* proximal enhancer. These control sequences are operably linked to a marker gene, the gene for enhanced green fluorescent protein (eGFP). The vector is designated pOct4-EGFP-PE (see Example 1). A polyadenylation signal sequence is also included in the vector.

In another embodiment of the invention, the control sequences of the *Oct4*/marker expression construct used to assay embryonic cells for germline competence comprise the *Oct4* distal enhancer (from *Bam*HI site at -4330 to the *Bam*HI site at -1220, Figure 1A) linked to the *Oct4* proximal promoter. For instance, to confirm that chicken blastodermal cells are able to contribute to germline tissues, they can be transfected with the expression vector pOct4-EGFP-DE shown in Figure 2B (Example 1). The vector pOct4-EGFP-DE lacks the proximal enhancer but instead contains a marker gene which is operably linked to the *Oct4* distal enhancer and *Oct4* proximal promoter.

In mice embryonic cells, the distal enhancer is active only in cells able to contribute to both somatic and germline tissues whereas the proximal enhancer

can, in some cases, be active in cells able to contribute to somatic but not germline tissues (Yeom *et al.*, 1996). However, in chicken blastodermal cells, the proximal enhancer appears to be specific for cells able to contribute to germline tissues as chicken blastodermal cells that were able to contribute to somatic but not germline tissues were not GFP-positive when transfected with pOct4-GFP-PE (Example 3, below). Thus, either the *Oct4* proximal enhancer or distal enhancer linked to the *Oct4* proximal promoter and marker gene can be used to determine the germline competence of chicken blastodermal cells.

In some cases, it may be desirable to use a combination of the distal enhancer with the proximal enhancer. In such a case, both the distal and the proximal enhancers and the promoter of the vector are all operably linked. Again, the promoter is the *Oct4* proximal promoter. For instance, the *Oct4* enhancer and promoter used in the *Oct4*/marker expression construct may be derived from the sequence ranging from approximately -4332 to approximately +29 of the mouse *Oct4* gene.

It is contemplated that additional control sequences from the *Oct4* gene may be used in the cells and methods of the present invention. Additional control sequences from the *Oct4* gene may optionally be used in combination with the *Oct4* promoter.

It is further contemplated that additional control sequences from genes other than *Oct4* which are substantially expressed only in cells that are germline-competent may be used in the cells and methods of the present invention. A gene which is substantially expressed only in cells which are germline-competent is a gene which shows at least an approximately 5-fold higher level of expression (as evidenced by protein levels) in cells which are germline-competent than in cells which are not germline-competent (under the same conditions). Preferably, the level of expression of the gene is at least about 10-fold higher in a germline-competent cell than in a cell which is not germline-competent. In still further preferred embodiments, the expression level of the gene is at least about 20- or

about 100 fold higher in cells which are germline-competent versus those which are not.

One of ordinary skill in the art will be able to readily establish if a given control sequence or combination of control sequences is suitable for use in the present invention. First, the gene from which the control sequence is derived must be substantially expressed only in cells which are germline-competent. This can be established by measuring and comparing protein expression levels in cells which are germline-competent versus the same type of cells which are not germline-competent. As a second step in establishing the ability of individual control sequences or combinations of control sequences to be used in the expression constructs of the present invention, the individual control sequences or combinations of control sequences from the gene are isolated and operably linked to a reporter gene in an expression vector using any of a number of techniques well known to those skilled in the art. The expression vector can then be transfected into cells and used to confirm that the ability to confer germline-competent specific expression is retained in the isolated control sequences or combinations of control sequences (for instance, see the specific example, Example 3, below).

The marker gene may be selected from any of the many marker genes known to those of ordinary skill in the art. The exact nature of the marker gene is not considered critical to the invention as long as the marker gene encodes a protein which can be readily detected via an existing technology. In preferred embodiments, the marker gene may be a reporter gene encoding green fluorescent protein (*GFP* gene), enhanced green fluorescent protein (*eGFP* gene), or β-galactosidase (*lacZ* gene). In alternative embodiments, the marker gene may encode yellow or blue fluorescent protein. Genes encoding chloramphenicol acetyltransferase (CAT) or β-lactamase (β-LA) provide additional possibilities. The use of a luciferase marker gene in the present invention is further

contemplated. Also, the marker gene may be a selectable marker gene such as the neomycin-resistance gene.

The nature of the detection of expression of the marker gene will, of course, be dependent upon the particular marker gene construct chosen. Detection of the common marker genes such as those listed herein are routine for one skilled in the art.

c) Introduction of the expression constructs of the invention into cells.

The specific nature of the method of transferring the expression constructs of the invention such as the *Oct4* marker expression construct into the target cells is not critical to the present invention. One of ordinary skill in the art can readily choose a method from the variety of known methods. The choice of gene transfer method will be dependent upon the specific animal of origin of the target cells as well as the size of the expression construct to be transferred. Numerous methods of gene transfer are presented here by way of example and are not to be construed as limiting. Also, different embodiments of the invention are exemplified below with respect to the *Oct4* marker expression construct. However, it is understood that such described preferred and alternative embodiments are also contemplated with respect to expression constructs of the invention which comprise control sequences from genes other than *Oct4* which are expressed substantially only in cells which are germline-competent. Some suitable methods of gene transfer are viral, others are non-viral. In a preferred embodiment, the *Oct4*/marker expression construct is delivered via non-viral means. Introduction of the expression vector into the target cell in the present invention may be either transient or stable. In a preferred embodiment, the *Oct4*/marker expression construct is introduced into the target cell by transient transfection.

Several non-viral methods for the transfer of expression constructs into the cells of avian or other animals are contemplated by the present invention. These include, but are not limited to, calcium phosphate precipitation, DEAE-dextran,

electroporation, direct microinjection, DNA-loaded liposomes, cell sonication, gene bombardment using high velocity microprojectiles, and receptor-mediated transfection.

In certain embodiments, the expression construct encoding the marker gene under control of the *Oct4* proximal promoter may be stably integrated into the genome of the cell. This integration may be in the cognate location and orientation via homologous recombination (gene replacement) or it may be integrated in a random, non-specific location (random gene insertion). In yet further embodiments, the nucleic acid may be stably maintained in the cell as a separate, episomal segment of DNA. Such nucleic acid segments or "episomes" encode sequences sufficient to permit maintenance and replication independent of or in synchronization with the host cell cycle. How the expression construct is delivered to a cell and where in the cell the nucleic acid remains is dependent on the type of expression construct employed.

In a particular embodiment of the invention, the expression construct may be entrapped in a liposome. Liposome-mediated nucleic acid delivery and expression of foreign DNA *in vitro* has been very successful and may likewise be used in the present invention. Various commercial approaches involving "lipofection" technology are also contemplated for use in the present invention.

In another embodiment of the invention, the *Oct4*/marker expression construct may simply consist of naked recombinant DNA or may be contained on a simple plasmid. Transfer of the construct may be performed by any method which physically or chemically permeabilizes the cell membrane. This is applicable particularly for transfer *in vitro*. Another embodiment of the invention for transferring a naked DNA expression construct into cells may involve particle bombardment.

In an alternative embodiment of the invention, viral infection of cells is used in order to deliver the *Oct4*/marker expression construct to a cell.

Adenovirus is particularly suitable for use as a gene transfer vector because of its mid-sized DNA genome, ease of manipulation, high titer, wide target-cell range, and high infectivity. For instance, adenoviruses can be used for delivery of transgenes to a wide variety of cell types. Human adenoviruses, such as those described in Fisher and Watanabe, *Cardiovascular Research*, 1996, 31:E86-95 and Lou *et al.*, *Journal of Orthopedic Research*, 1996, 14:513-517, both herein incorporated by reference, have been shown to be efficient means of introducing genes into chicken cells. The human adenovirus vectors described in Fisher and Watanabe and Lou *et al.* are replication-deficient and do not integrate into DNA. Thus, the transgene persists only transiently in cells and long-term expression is not expected.

As an alternative to adenoviruses and for long-term expression, retroviruses may be used as the delivery vehicles. For instance, in one embodiment of the invention, the target cells are avian and the gene delivery vehicle is a replication-deficient retrovirus based on the avian leukosis virus (ALV) that has been used to produce germline transgenic Brown Leghorns (Cosset *et al.*, *Journal of Virology*, 1991, 65:3388-94; Thoraval *et al.*, *Transgenic Research*, 1995, 4:369-377).

Alternatively, the VSV-G envelope protein of the vesicular stomatitis virus (VSV) pantropic retrovirus can be used to deliver the expression construct to an avian cell. The VSV pantropic vector, LZRNL-G, encodes β-galactosidase under control of the Moloney murine leukemia virus and is packaged with the G envelope glycoprotein (G-protein) of the vesicular stomatitis virus (VSV-G) (see Burns *et al.*, *Proc. Natl. Acad. Sci. USA*, 1993, 90:8033-8037). This transducing particle has been shown to integrate into the genomes of a variety of species, including chickens (Burns *et al.*, *Proc. Natl. Acad. Sci. USA*, 1993, 90:8033-8037; Lin *et al.*, *Science*, 1994, 265:666-669; Yee *et al.*, *Methods in Cell Biology*, 1994, 43:99-112).

It will be apparent to one of ordinary skill in the art that retroviruses other than the ones specifically mentioned here will be useful in the transgenesis

methods described herein. Both replication-defective and replication-competent retroviruses can be used for the present invention, although replication-defective retroviruses are preferred.

Other viral vectors may be employed as expression constructs in the present invention. Vectors derived from viruses such as adeno-associated viruses, vaccinia viruses, canary pox viruses, bovine papilloma viruses, lentiviruses, and herpes viruses may also be employed.

d) Use of the expression construct to assay for germline-competent avian cells.

The present invention provides for methods of using the *Oct4*/marker expression construct and other expression constructs to assay for the germline competence of avian cells. The cell which is assayed for germline competence may be an avian stem cell or an avian germ cell. In preferred embodiments, the avian cells are embryonic cells or cells derived from embryonic cells.

In a particularly preferred embodiment, the avian embryonic cells are chicken blastodermal cells. These cells may optionally have been freshly isolated from an avian blastoderm, such as, but not limited to, a chicken blastoderm at a stage of development from about stage IV to about stage XII. Preferably, the chicken blastoderm is at a stage of development from about stage VIII to about stage XII, and most preferably at about stage X. These blastodermal cells, although isolated, may be uncultured when assayed for germline competence. In an alternative embodiment, the avian embryonic cells being assayed may still be in an intact avian embryo, such as a chicken blastoderm. In a preferred embodiment, the avian embryonic cells which are to be assayed for germline competence are cultured cells. For instance, the cells may be cultured blastodermal cells such as, but not limited to, cultured chicken blastodermal cells. The avian embryonic cells used in the invention may be embryonic stem cells or

embryonic germ cells, either in culture or not in culture. Primordial germ cells may also be assayed using the methods of the present invention.

The avian cells may be derived from any type of avian animal. In a preferred embodiment, the avian animal is a chicken. The chicken may be any of the various known strains of *Gallus gallus* including, but not limited to, White Leghorn, Brown Leghorn, Barred -Rock, Sussex, New Hampshire, Rhode Island, Ausstralorp, Minorca, Amrox, California Gray, or Italian Partidge-Colored. Alternatively, the avian animal may be selected from the group consisting of turkeys, pheasants, quails, duck, geese, and other poultry commonly bred in commercial quantities.

In one embodiment of the present invention, a method for identifying avian cells which can give rise to germline tissue comprises the following steps: first, introducing into avian cells an expression construct containing a marker gene operably linked to control sequences sufficient for effecting expression of the marker gene in germline-competent cells, wherein the control sequences comprise at least one control sequence from a gene which is substantially expressed only in cells which are germline-competent; and second, assaying for expression of the marker gene in the cells, wherein the expression of the marker gene in a particular cell indicates that that particular cell can give rise to germline tissue. In a preferred embodiment, the expression construct used in the method comprises an *Oct4* proximal promoter and a marker gene, all operably linked. The ability of the *Oct4*/marker expression construct to serve as a marker for germline competence in avian cells is demonstrated in Example 3 and Figure 4.

Possible methods of introducing the *Oct4*/marker expression constructs into the avian cells include, but are not limited to those listed above. However, transient transfection methods are often both sufficient and preferred when the targeted avian cells are recent explants or are in culture. If the cells are to be assayed *in vivo*, such as within an avian embryo, then viral delivery methods are preferred.

In another embodiment, a method of identifying avian embryonic cells that can contribute to germline tissues of recipient embryos is provided. This method involves as a first step introducing an expression construct comprising the marker gene and the control sequences at least partially derived from a gene that is substantially expressed only in cells which are germline-competent into an avian embryonic cell. Preferably, the expression construct is the *Oct4*/marker expression construct. In a second step, the method comprises assaying for expression of the marker gene in the cell. In such a method, the expression of the marker gene in the cell indicates that that particular cell can contribute to germline tissues of a recipient embryo upon injection into that recipient embryo.

For instance, to determine if a given set of cultured chicken blastodermal cells can give rise to germline chimeras, one transfects a plasmid containing an *Oct4*/marker expression construct in which the *Oct4* proximal promoter is operably linked to *GFP* into cultured chicken blastodermal cells the day before injections into recipient embryos are to be carried out. The next day one visualizes the marker gene in the cells by exciting GFP with UV light at the appropriate wavelength and viewing the fluorescing cells with the correct emission filter. If the cells are fluorescing, then the cells can and will contribute to germline tissues. If the cells are not fluorescing, then the cells may still contribute to somatic tissues, i.e. feathers, but not germline tissues.

Alternatively, if the marker gene of the *Oct4*/marker expression construct is a selectable marker gene, assaying for expression of the marker gene may involve subjecting the cells to conditions in which the expression of the selectable marker gene by an individual cell is required for survival of that individual cell. For instance, if the marker gene is the neomycin-resistance gene, expression of the marker gene and hence, germline competence, can be determined by adding G418 to the cell culture media. In this selection assay, only those cells which survive are germline-competent.

The present invention also provides for a method of creating chimeric avians, including, but not limited to, germline chimeras. This method entails first introducing into avian embryonic cells an expression construct which comprises a marker gene operably linked to control sequences sufficient for effecting expression of the marker gene in germline-competent cells, wherein the control sequences comprise at least one control sequence from a gene which is substantially expressed only in cells which are germline-competent. For instance, the control sequences of the expression construct preferably comprise a proximal promoter from *Oct4* enhancer. Next, avian embryonic cells which express the marker gene sequence are identified. These cells (typically about one hundred or more) are then transferred to a recipient avian embryo, where the genome of the recipient avian embryo differs from that of the transferred cell. In a preferred embodiment, at least some of the cells which are transferred to the recipient avian embryo contribute to its avian embryo. In a final step, the recipient embryo is allowed to develop at least until it hatches.

Methods of producing a transgenic avian or modifying the germline of an avian animal are further provided by the present invention. These methods involve introducing a transgene of interest into one or more avian embryonic cells. Either before, after, or at the same time as the transgene of interest is introduced into the cells, an expression construct comprising a marker gene operably linked to control sequences sufficient for expression of the marker gene in germline-competent cells is introduced into the cells. At least one of the control sequences must be a control sequence of a gene which is substantially expressed only in germline-competent cells. Preferably, the *Oct4*/marker expression construct is used. Avian embryonic cells which comprise the transgene of interest and which also express the marker gene sequence are identified. The cells which are identified as expressing the marker gene sequence are then transferred to an avian embryo, which is subsequently allowed to develop to hatch. In a preferred embodiment, at least some of the transferred cells contribute to the germline tissue

of the recipient embryo and the hatched avian animal carries the transgene in its germline tissue.

Preferably, the *Oct4*/marker expression construct or other expression construct suitable for indicating germline-competence is delivered to the cell after the transgene of interest has been introduced to the cell. In many cases, the success of the first transfection is established prior to performing the assay for germline competence. For instance, one may wish to check if the transgene has been integrated in the correct position and orientation before proceeding with transfection of the *Oct4*/marker expression construct into the cells. Introducing the *Oct4*/marker expression construct to the transgenic cells via transient transfection shortly before transferring the cells to the embryo is preferred.

In an alternative preferred embodiment, the Oct4/marker expression construct and the transgene of interest are cotransfected into the cell.

Still, other possibilities exist for alternative timings for introducing the *Oct4*/marker expression construct and the transgene into the cell. For instance, the *Oct4*/marker expression cassette may be transfected first and the other expression construct second. This transfection order would be practical only in some cases, for instance when the *Oct4*/marker expression construct integrates into the genome of the cell or replicates episomally in a stable manner. When the marker gene is a selectable marker gene or encodes destabilized eGFP (for instance, d2EGFP from Clontech), then transfecting with the *Oct4*/marker expression construct first would also be suitable. Alternatively, in some cases, both the *Oct4*/marker expression construct and the other transgene may be included on a single vector which is introduced into the cell.

The aforementioned method is particularly advantageous when the avian embryonic cells which ultimately serve as donors to the recipient embryo have been cultured, since most avian embryonic cells lose germline competence over time in culture and become unable to contribute to germline tissue.

The exact nature of the transgene of interest is not critical to the present invention. Many operably linked combinations of a promoter (optionally in combination with other control sequences) which is active in avian animals and a coding sequence may be employed in the present invention. The coding sequence of the transgene may encode an exogenous protein or peptide. Alternatively, the coding sequence may encode an antisense RNA molecule or a ribozyme. The promoter on the transgene may be constitutive, tissue-specific, or inducible. The expression vectors described in WO99/19472, herein incorporated by reference, can optionally be used as transgenes in the present invention.

In preferred embodiments, introduction of the transgene of interest into the germline-competent avian embryonic cell results in integration of the transgene of interest into the genome of the cell. Optionally, one of the following methods may be used to achieve integration of the transgene into the host cell genome: random gene insertion, gene targeting via homologous recombination , and retroviral delivery.

To effect random gene insertion into the genome of the host cell, a transgene addition vector may be used, such as the linearized plasmid described in Example 4, below. A transgene addition vector is an expression vector which typically comprises a promoter operably linked to a coding sequence of interest and a transcription termination signal. Additional control sequences may also be present. The transgene addition vector also typically comprises a marker gene (also operably linked to a promoter and transcription termination signal) which allows for the identification of cells which have integrated the vector. Transgene addition vectors are typically linearized.

If gene targeting via homologous recombination is desired, the transgene of interest may optionally take the form of a gene-targeting vector such as a promoter-less minigene. The promoter-less minigene may comprise a coding sequence of interest, at least one marker gene which is operably linked to a constitutive promoter and can be used for identifying cells which have integrated

the targeting vector, and targeting nucleic acid sequences which correspond to sequences flanking the point of insertion in the target gene, wherein the targeting nucleic acid sequences direct insertion of the targeting vector into the target gene. Typically, the designed point of insertion is an untranslated region of the target gene. In alternative embodiments, a targeting vector may be used which further comprises a promoter that is operably linked to the coding sequence.

Where integration is not desired, an artificial chromosome which is stable in avians may alternatively be used to carry the transgene of interest. In general, the transgene of interest may be contained within any suitable vector or delivery vehicle such as those described above in conjunction with the *Oct4*/marker expression construct. One of ordinary skill in the art will be able to readily choose an appropriate expression construct/delivery method combination suitable for the given transgene and desired length of the expression period.

Methods for transferring an avian embryonic cell, in some cases following genetic manipulations to an avian embryo, are well known to those skilled in the art. Typically, portions of the shell and outer shell membranes are removed from the recipient embryo's egg to expose the embryo. For example, in one embodiment an opening about 5mm in diameter is made in the side of an egg, normally by the use of a drilling tool fitted with an abrasive rotating tip which can drill a hole in the egg shell without damaging the underlying shell membrane. The membrane is then cut out by use of a scalpel. The genetically altered embryonic cells are then injected into the egg containing the embryo. The cell or cells may be injected into the yolk sac or onto the chorioallantoic membrane, preferably into the subgerminal cavity, and preferably during early embryonic development such as prior to day 2 or 3 of incubation, and most preferably prior to day 1 of incubation. Examples of methods for transferring avian cells to recipient embryos can be found in the following references, all of which are herein incorporated by reference: Watanabe *et al.*, *Development*, 1992, 114:331-338; Fraser *et al.*, *Int. J. Devel. Biol.*, 1993, 37:381-385; Thoraval *et al.*, *Poultry Sci.*, 1994, 73:1897-1905;

Carsience *et al.*, *Development*, 1993, 117:669-675; Petitte *et al.*, 1990, 108:185-189; U.S. Patent No. 5,656,479; Brazolot *et al.*, *Molecular Reproduction and Development*, 1991, 30:304-312; and U.S. Patent No. 5, 897, 998.

The step of allowing the avian embryo to which the germline-competent cell has been transferred to develop to hatch is routine for one of ordinary skill in the art. For instance, if the egg has been windowed, then the opening in the egg is typically resealed with shell membrane and a sealing material, preferably glue or paraffin. The sealed egg is then incubated first at 37.5°C for a few days, and then at 37°C until hatch.

Many possible applications of the methods of producing a germline-modified transgenic avian animal exist. For instance, in one embodiment, the present invention can be used to create a line of germline-modified transgenic chickens which express exogenous proteins in their oviducts and deposit those exogenous proteins in their eggs. In such a case, chicken blastodermal cells are initially transformed by a retroviral particle comprising an avian leukosis virus pseudotyped with the G-envelope glycoprotein of vesicular stomatitus virus (VSV). This retroviral vector carries a transgene comprising a minimal ovalbumin promoter operably linked to an exogenous coding sequence, such as the human growth hormone gene, and signal peptide. (Alternatively, a nonviral vector could be used for the transgene.) The *Oct4*/marker expression construct is then used to identify which of the transformed chicken blastodermal cells are germline-competent and can contribute to germline tissue. The transgenic chicken blastodermal cells which are to be tested for germline competence are transiently transfected via calcium phosphate precipitation with plasmids carrying the *Oct4/EGFP* expression construct. Sorting by FACS identifies the germline-competent cells. The germline-competent cells which carry the human growth hormone transgene are then transferred to a stage X blastoderm in a windowed egg.

Some preferred procedures for the production of transgenic chickens using chicken blastodermal cells and the *Oct4*/marker expression constructs of the present invention are described in the specific example, Example 4, below.

e) Screens for cell culture conditions and cellular factors which promote the continued germline competence of cells in culture.

An alternative embodiment of the invention provides for a method of screening culture conditions for those which allow for the long-term germline competence of animal cells in cell culture. In this embodiment, a population of cells is subjected to selected culture conditions for a selected period of time. After the selected period of time has elapsed, or alternatively, near the end of the time period, an *Oct4*/marker expression construct is introduced into the cell or population of cells. The cell or population of cells is then assayed for expression of the marker gene. Expression of the marker gene by cells or, preferably, a significant portion of the cells, in the population indicates that those cells have remained germline-competent under the selected cell culture conditions for the selected period of time. If the cells have remained germline-competent for an unexpectedly long period of time, then the selected cell culture conditions may contain a factor which promotes the maintenance germline competence in chicken blastodermal cells.

Alternatively, instead of the *Oct4*/marker expression construct, an expression construct is used in the method which comprises a marker gene operably linked to any control sequences sufficient for effecting expression of the marker gene in germline-competent cells, wherein the control sequences comprise at least one control sequence from a gene which is expressed only in cells which are germline-competent.

Typically, the ability of the tested cell culture conditions to promote continued germline competence will be compared against the ability of traditional or control culture conditions to promote germline competence of cells over the

same period of time. In a preferred embodiment, new culture conditions will be compared in parallel with control conditions.

In a preferred embodiment of the invention, a plurality of populations of cells, each subjected to a different set of culture conditions, are screened in parallel.

The nature of the different cell conditions which may be screened using the present invention can vary greatly. In a preferred embodiment, changes in cell culture media composition are screened. Changes in the cell culture media can optionally comprise changes in amounts or proportions of the different components of the cell culture media. Preferably, however, a variety of newly added factors, such as growth factors, are tested. (Some factors, such as fibroblast growth factor, leukemia inhibitory factor, and soluble Steel factor, have been previously identified as being advantageous in stem cell culture (U.S. Patent No. 5,670,372)). The factors may optionally comprise either isolated natural proteins or peptides or recombinant proteins or peptides. The recombinant proteins may be identical to the naturally occurring entity or they may have been altered, for instance, truncated or mutated. Alternatively, the added factors may be small molecules, such as those generated by combinatorial synthetic methods or produced by phage display. In alternative embodiments, other aspects of cell culture are screened, such as temperature or mechanical techniques. Cell culture conditions which can be screened by methods of the present invention are understood not to be limited to those listed here. The cell culture conditions to be screened may also comprise any combination of those listed here.

In one embodiment, the cells used in the screen are stem cells or germ cells, such as embryonic stem cells, embryonic germ cells, or primordial germ cells. In a preferred embodiment, the cells used in the screening method are avian cells, such as chicken blastodermal cells. However, this screening method is applicable to a wide variety of cell types and is not limited to just avian embryonic cells or even avian cells in general. Since *Oct4* is known to be expressed in mice and

other mammals, the invention is applicable to screens for improved culture conditions useful in maintaining the germline competence of cells of murine animals or other mammals such as humans, sheep, goats, horses, rabbits, cattle, and pigs. Since it has now been demonstrated herein that the mouse *Oct4* promoter is also active in non-mammals (see Examples 2 and 3, Figures 3 and 4), the targeted cells need not be limited to either birds or mammals. Thus, the cells used in the screen can optionally be those of other vertebrates such as fish.

In one embodiment, the invention method is used to screen factors for those which help maintain the germline competence of chicken blastodermal cells in culture after about 4 days. For instance, the method may be used to screen factors which help maintain the germline competence of chicken blastodermal cells from about 4 to about 8 days in culture. The method essentially assays the activity of the *Oct4*/marker expression construct in transfected chicken blastodermal cells after 4 days of culture. Factors identified by such a screen would be extremely useful for long-term culture of germline-competent chicken blastodermal cells. Culture conditions modified by addition of the identified factor enables successful use of new sophisticated genetic methods to modify the chicken genome, including random gene insertion, gene targeting, and the use of artificial chromosomes. The *Oct4* assay can also be used to screen for culture conditions that prevent the loss over extended periods of time (substantially greater than 4 days) of the ability of chicken blastodermal cells to contribute to germline tissues. For instance, the present invention can be used to screen for culture conditions that prevent the loss of germline competence in chicken blastodermal cell in culture over one week, two weeks, three weeks, one month, two months, or more.

For example, in one embodiment, chicken blastodermal cells isolated from stage X embryos are cultured in wells of a 96-well plate on a feeder cell line known to maintain the growth of chicken blastodermal cells. To each well is added a different secreted or soluble factor or a unique set of secreted factors from an expression library (Wong, *Genetic Engineering*, 1990, 12:297-316). After

about 4-8 days of culture, cells are transfected with the *Oct4*/marker expression construct. The next day the marker gene is visualized. One then scans the wells for cells that are expressing the marker gene, indicating that the *Oct4* promoter is still active and the cells are able to contribute to germline tissues. A marker gene-positive well indicates that a factor was present that maintains the germline competence of chicken blastodermal cells. That factor could then be isolated from the expression library, produced in large amounts and used routinely for chicken blastodermal cell culture.

Some specific procedures for screening for factors useful in maintaining the germline competence of chicken blastodermal cells are outlined in the specific example, Example 5, below.

One advantage of the present invention is that the *Oct4*/marker assay and related assays are amenable to adaptation to high throughput assays for factor screening and identification of culture conditions which allow long-term culture of cells, such as germline-competent chicken blastodermal cells. The ability to adapt a stringent, sensitive assay to reproduction in 96-well plates or some high-capacity format is an advantage of the present invention. The production of cell cultures demonstrating long-term maintenance of germline competence is contemplated by the present invention.

f) Examples

The following specific examples are intended to illustrate the invention and should not be construed as limiting the scope of the claims.

Example 1. *Oct4* promoter/marker gene vector construction.

The vector pOct4-GFP-PE (Figure 2A) was created by first assembling the *Hin*dIII/*Not*I EGFP-encoding fragment from pEGFP (Clontech) and the *Not*I/*Bam*HI polyadenylation signal fragment from pRC/CMV (Clontech) into the *Bam*HI site of pBluescript KS+ such that the *Cla*I and *Eco*RI sites of pBluescript's

polylinker resides 5' of the *eGFP* open reading frame (ORF). The proximal enhancer (PE) and proximal promoter of the *Oct4* promoter was isolated by PCR amplification from GOF-6 (Yeom *et al.*, 1996) using oligos with the following sequences: AATTAACCCTCACTAAAGGG (SEQ ID NO:2) and GGCCCGGGAATTCGGAAAGACGGCTCACCT (SEQ ID NO:3). The PCR product was cut with *ClaI* and *Eco*RI and cloned into *Cla*I and *Eco*RI sites of the intermediate vector described above.

The vector pOct4-GFP-DE (Figure 2B) was created by replacing the *Hind*III/*Bst*EII region of pOct4-GFP-PE with the *Bam*HI-*Bam*HI distal enhancer (DE)-containing fragment of GOF-9 (Yeom *et al.*, 1996).

pOct4-EGFP-DE was constructed by digestion of pOct4-EGFP-PE (aka pOct4-GFP-PE with *Bst*EII and *Hind*III. The ends were polished by addition of the Klenow fragment of DNA polymerase I and dNTPS. The vector was religated to itself. See Table 1 for the results of transfection experiments with these vectors.

Example 2. The activity of the *Oct4*/marker expression construct in chicken blastodermal cells decreases over time in culture.

Chicken blastodermal cells were collected from stage X blastoderms as described in Example 4, below. Some chicken blastodermal cells were transfected with the Oct4-GFP-PE expression vector (see Example 1 for Oct4-GFP-PE map and Example 4 for transfection protocol) and then cultured for a total of 24 hours (see Example 4 for culture conditions). A photograph of these cells is shown in Figure 3A. The fluorescent image of the same cells is shown in Figure 3B. In another experiment, the chicken blastodermal cells from stage X blastoderms were cultured for 6 days before being transfected with the *Oct4*/marker expression construct (Oct4-GFP-PE) (see Example 4 for culture and transfection protocols). After culturing the cells for an additional 24 hours (total culture time equals 7 days), the cells were photographed (Figure 3C). The fluorescent image of the same cells is shown in Figure 3D. The images show markedly decreased expression of GFP in the cells which have been subjected to longer periods of time

in culture (7 days) compared to those which were subjected to only 24 hours in culture.

Example 3. *Oct4* activity in chicken blastodermal cells correlates with germline competence.

Chicken blastodermal cells were cultured on fibroblasts isolated from whole embryos using standard chicken blastodermal cell culture media as described in Example 4, below. (In some experiments, factors were added to the media in an attempt to maintain totipotency, but the addition of these factors did not affect the results shown in Figure 4.) Chicken blastodermal cells were cultured for various times and transfected with the expression vector pOct4-GFP-PE (transfections were performed as described in Example 4). The next day the percent of GFP-positive cells was determined ("% Oct4-GFP-PE expression" in Figure 4). The GFP-positive chicken blastodermal cells were then immediately harvested and injected into γ-irradiated White Leghorn recipient embryos as described in the Detailed Description of the Invention, above. The recipient embryos were then allowed to hatch. Hatched chicks were scored for the presence or absence of black feathers; the former number divided by the total number of chicks gave the percent of somatic chimeras. All males were raised to sexual maturity and bred to Barred Rock females. Only Barred Rock offspring (and not White Leghorn offspring) originated from injected cultured chicken blastodermal cells that populated the chimera's germ tissues. The data points marked "% germline chimeras" in Figure 4 indicate the percentage of those males that were able to transmit the Barred Rock black feather marker, and thus the genetic material of the cultured/injected chicken blastodermal cells, to their offspring.

The results of the experiments are shown in Figure 4. Data points for "% germline chimeras" represent at least 3 separate experiments for each period of culture, except for the data point at 2 days which represents 2 separate experiments. Germline transmission was not determined for days 4, 16 and 23.

Note that the percentage of Oct4-GFP-PE expression correlates well with the percentage of germline chimeras but does not correlate with the percentage of somatic chimeras. Therefore, the *Oct4*/marker gene construct is a good predictor of the ability of cultured chicken blastodermal cells to contribute to germ tissues, but not to somatic tissues.

Example 4. Production of transgenic chickens via chicken blastodermal cell culture and *Oct4* screening.

Culturing chicken blastodermal cells. Chicken blastodermal cells are isolated from freshly laid Barred Rock eggs as described in (Carsience *et al.*, 1993). Using filter paper rings, the blastoderms are collected into Dulbecco's phosphate buffered saline containing 1g/L D-glucose (PBS-G; Gibco-BRL). The PBS-G is replaced with cold Dulbecco's calcium- and magnesium-free phosphate buffered saline (PBS-CMF, Gibco-BRL) (1 mL/tube) and the cells are incubated on ice for 10 minutes. The PBS-CMF solution is replaced with 0.05% trypsin/0.02% EDTA (t/e) solution and the cells are then incubated on ice for 10 minutes. Next, the t/e solution is replaced with Dulbecco Modified Eagle's Medium (DMEM) (Gibco BRL) plus 10% fetal bovine serum (FBS). The sample is vortexed briefly (~speed 3) and then centrifuged for 5 minutes at 500 rpm (60 x g) at 4°C. After aspirating the DMEM, the cell pellets are gently resuspended and pooled into 1 tube in about 1 mL of DMEM plus 10% FBS. The cells are counted. The chicken blastodermal cells are plated on gelatin-coated plates or on feeders at ~1.8×10^5 cells/35mm well. (Because the process is scalable, alternatively, other formats such as 96-well plates may be used.) The cells are plated either directly onto tissue culture plates or onto a feeder cell line that promotes proliferation and germline competence. The media can be any media that promotes proliferation and germline competence of chicken blastodermal cells. Examples of media constituents are: conditioned media, media supplemented with one or more soluble factors, media supplemented with one or more animal serum/sera.

Ideally chicken blastodermal cells are cultured on fibroblasts isolated from whole embryos (termed WEFs). WEFs are isolated as described in (Kuwana *et al., Int. J. Dev. Biol.*, 40:1061-4, 1996) with the following differences. Fertile chicken eggs are incubated for approximately 65 hours (embryos should reach stage 16 or 17; ~30 somites). Embryos are collected using filter paper rings as per the chicken blastodermal cells. The inverted embryo is briefly washed in PBS-G (Gibco-BRL) then placed in an empty petri dish (making sure embryos remain wet throughout the procedure). Next, embryos are removed by cutting through the outer membrane and placing them in a 6 cm dish containing PBS-G. Embryos are washed 3 times with PBS-G to remove debris. The embryos are aseptically washed with PBS-CMF (Gibco-BRL) plus 0.02% EDTA, then incubated in fresh PBS-CMF plus 0.02% EDTA at 4°C with gentle shaking for 30 minutes. The solution is removed and the embryos incubated in 0.5% trypsin solution in PBS (no EDTA) at 37°C for 3 minutes. The solution is pipetted up and down 10 times using a Pasteur pipette 'seasoned' with chicken serum. The embryos are incubated for a further 2 minutes, then triturated as before. The embryos are incubated another 2 minutes, then 1 mL of chicken serum is added and the solutions is triturated again. The cell suspension is added to 12 mL of α-MEM (complete) (α-MEM with 2.2 g/L $NaHCO_3$, 2.52 g/L N-[2-hydroxyethyl]piperazine-N'-3-propanesulfonic acid (EPPS) 0.18 g/L D-glucose, pH 8.0, 5% fetal bovine serum, 5% heat inactivated chick serum, 50 μM β-mercaptoethanol, 0.2 mM L-glutamine, 1X penicillin/streptomycin) and centrifuged at 600 rpm (speed 4) for 5 minutes. The cell pellet is then resuspended in 1.0 mL α-MEM (complete). Each embryo yields ~1.2×10^5 cells.

Transfection of cultured chicken blastodermal cells with a transgene. Prior to or after plating, chicken blastodermal cells are transfected with a linearized plasmid carrying the desired transgene. The transgene includes elements that result in modification of the chicken as well as elements that facilitate genetic

modification of chicken blastodermal cells. For instance the transgene may contain elements that direct expression of a protein in the magnum of hens. Preferably, the transgene also contains elements that allow one to screen or select for genetically modified cells.

Cultured chicken blastodermal cells are transfected with a linearized plasmid carrying a transgene which contains a promoter that is active in chicken blastodermal cells linked to the neomycin (G418) resistance gene (*neo*^{r}) and a transcription termination signal. An exogenous gene of interest can also be operably linked to the promoter on the linearized plasmid and separated from *neo*^{r} by an internal ribosome entry site (IRES) element. The cells are transfected with the transgene prior to plating in a 35 mm well (6-well plate). Chicken blastodermal cells from stage X blastoderms are collected and dispersed. The cells are then suspended in OptiMEM (Gibco-BRL). In a 13 x 75mm polystyrene culture tube, 4 μg of DNA are mixed with 100 μL of OptiMEM per well. In a separate tube, 16 μg of Lipofectin (Gibco-BRL) is mixed with 100 μL of OptiMEM. The DNA solution and the Lipofectin solution are then mixed together and incubated at room temperature for 30 minutes. 1.8×10^5 cells per well are plated in 100 μL OptiMEM. The DNA/Liposome mixture is overlaid on the cells, 600 μl OptiMEM added, and the suspension swirled to mix thoroughly. The cells are incubated for 3.5 hours at 37°C in 5% CO_2/95% air. The volume of each well is brought up to 2 mL by addition of warm culture medium. The cells are incubated overnight, and then the medium is changed. The procedure for cells that have already attached to the culture vessel involves washing the attached cells twice with OptiMEM prior to transfection, and after the 3.5 hour incubation, the medium is aspirated and replaced with warm medium appropriate for the cells.

The cells are cultured for 4 to 12 days in the presence of 50 to 400 μg/ml G418. The G418 concentration will depend on the sensitivity of chicken blastodermal cells. The time of culture depends on when neo^r colonies are visible

and is usually proportional to the rate of cell division. Faster cell division rates give rise to shorter selection periods and vice versa. During the culture period, cells may be passaged and replated at cell densities optimal for proliferation and visualization of neo^r cells.

If the cells are to be passaged, the media is removed and the cells washed with PBS-CMF. The cells are then incubated for 15 minutes at room temperature in PBS-CMF containing 0.02% EDTA. The cells begin to loosen from the plate. The PBS is carefully aspirated (it is advisable to use a pipet for this purpose rather than the suction flask as cells are only loosely attached at this point). The solution is replaced with prewarmed 0.25% trypsin in PBS-CMF plus 0.02% EDTA. The cells are incubated at 37°C for 5 minutes. DMEM plus 10 % FBS is added and the cell suspension transferred to a 15 mL tube. The cells are centrifuged at 500 rpm (60 x g) for 5 minutes. The cell pellet is aspirated and resuspended in DMEM plus 10% FBS using a P1000 tip. Most of the feeder cells will remain in stringy clumps. Clumps are removed with the pipet tip leaving the remaining cell suspension (mostly chicken blastodermal cells) behind. Cells are then replated on a fresh feeder layer and passaged at a ratio of 1:2.

When neo^r colonies are visible, two options are available.

Assay for germline competence of transfected chicken blastodermal cells. First, if germline competence using a given set of culture conditions can be maintained for no longer than the time it takes for G418 selection, then the cells are tested with the *Oct4* assay for germline competence and injected into recipient embryos. I n this case, one assumes that the neo^r colonies are stably transfected and, for the most part, represent integration of the transgene into the chicken genome. If culture conditions allow, individual colonies are isolated, passaged and analyzed for the desired genetic modification. A longer period of culture allows one to screen neo^r chicken blastodermal cell colonies for the desired genetic modification. The length of time available for culture is determined

empirically prior to start of the experiment. During the experiment, samples of the cells can be tested for germline competence using the *Oct4* assay described below.

The day before injection, chicken blastodermal cells are transfected with pOct4-EGFP-PE, or pOct4-EGFP-DE, or pOct4-EGFP-PP using the chicken blastodermal cell transfection protocol described above. The next day one visualizes GFP expression by exposing the cells to UV light at the appropriate wavelength and viewing the fluorescing cells under a microscope with the correct filter. For enhanced GFP (EGFP)(Clontech), the excitation and emission wavelengths are 488 and 507 nm, respectively. Only cells that are GFP-positive are selected for injection into recipient embryos since only these cells will be able to contribute to germline tissues. Two methods can be used to select GFP-positive cells. First, if entire colonies or a significant proportion thereof are GFP-positive, then individual colonies can be scraped off the plates using hairpin loops and transferred to a collection tube using a finely-drawn needle and mouth pipetting. If only a small fraction of each neo^r colony contains GFP-positive cells, then those cells should be selected by fluorescence-activated cell sorting (FACS).

For FACS, chicken blastodermal cells are dispersed into a single cell suspension essentially as described for passaging. The cells are resuspended in OptiMEM plus penicillin/streptomycin to give a concentration of 10^6 cells/mL. If necessary, the cells are passed through a 40 μm nylon mesh prior to sorting. GFP-positive cells are immediately separated from negative cells by FACS, so that the cells do not clump back together. The start-up procedure of the flow cytometer is performed according to the manufacturer's instructions. The following protocol describes that used to sort chicken blastodermal cells according to the expression of GFP using a Coulter EPICS Elite. Using other fluorofores or instruments may require different settings or procedures. The cytometer is set to sort 3 droplets per cell. PMT settings are set such that only 2-3% of non-transfected control chicken blastodermal cells show fluorescence above the first decade (488 nm excitation, 507 nm emission filter sets). Transfected chicken blastodermal cells are

introduced and set sort gates are set to collect cells exhibiting fluorescence above the control population in one collection tube, the "positive" pool, while collecting the "negative pool" in another tube. The collection tubes are filled with enough medium that the deflected cells land on the surface of the liquid.

GFP-positive cells are concentrated by low speed centrifugation and injected into recipient White Leghorn embryos as described earlier in the Detailed Description of the Invention. Hatched chicks are grown to sexual maturity and bred. Typically one expects that a significant proportion of the putative chimeras contain the Barred Rock (BR) feather marker, indicating a successful experiment so far. However, one should not always expect to obtain somatic chimeras because the GFP positive cells selected for injections may only be able to contribute to a small subset of tissues, which may include germline tissues but exclude ectodermal tissues, for instance. All male chicks are bred and only BR progeny are saved for further testing. These chicks arose directly from gametes that originated from GFP-positive chicken blastodermal cells and thus should contain any genetic modification that was carried by the chicken blastodermal cells.

The BR chicks are analyzed to determine the nature of the genetic modification. Southern blots are used to determine that the transgene is intact and integrated into the chicken's genome. Fluorescent *in situ* hybridization (FISH) is also used to confirm the chromosome into which the transgene has integrated. The transgene functionality is determined by assaying for the expected phenotype. For instance, if the transgene is designed to express a secreted protein specifically in the magnum of laying hens, then the egg white of transgenic hens are examined for the presence of the desired protein.

Example 5. Screening for factors that maintain the germline competence of cultured chicken blastodermal cells.

Chicken blastodermal cells are isolated from freshly laid eggs as described above. The cells are seeded onto 96-well plates. The cells are plated either directly onto gelatin-coated wells or onto a feeder cell line that promotes proliferation and germline competence. The medium is any that promotes proliferation of chicken blastodermal cells but is ideally DMEM with 10% FBS. The number of wells plated is determined by the number of factors or culture conditions to be tested. If only a few conditions are to be tested, larger wells can be used. At a minimum, culture conditions should be tested in duplicate. Thus if 96 factors are to be tested, two 96 well plates should be plated with chicken blastodermal cells. Extra wells are saved for transfection controls. An acceptable transfection control is transfection of several wells with a plasmid containing a constitutive promoter linked to the same marker gene as in the *Oct4*/marker gene expression construct; in this case, the marker gene is GFP. From 1,000 to 10,000 chicken blastodermal cells are plated per well with 3,000 being the optimum number.

Each pair of wells in the duplicate 96-well plates contains medium that is unique in composition. For instance, a number of different media containing various combinations of animal sera are made up and added to each pair of wells. Alternatively, the same media is added to all of the wells and then unique media

supplements are added to each pair of wells. Prior to seeding the chicken blastodermal cells, a 96-well plate can be set up that contains a unique soluble factor in each well. This plate is designated the master plate and facilitates the distribution of a large number of factors to the chicken blastodermal cells.

If the soluble factors are pure or relatively concentrated, they are diluted in the master plate such that when 1 to 10 μl of that factor is added to the chicken blastodermal cell 96-well plate, the factor is at a final concentration that is likely to result in bioactivity. Alternatively, factor libraries can be used of which each well contains conditioned media from cells (typically COS cells) that were transformed with a cDNA expression vector that expresses a unique secreted protein. Thus each well of the 96-well plate contains media, each of which contains a different factor. These libraries are designed to identify factors which will elicit a desired phenotype or biological activity in a particular cell type, a process called expression cloning (see Wong, 1990). The concentration of factors in the conditioned media is 0.1 to 0.5 μg per ml. Most cytokines and other soluble factors exert their biological activity in the picomolar level. For a protein of approximately 10 kD, 1 picomolar is 10 pg per ml. Thus, the conditioned media is added to the chicken blastodermal cell media such that the final dilution is 1 in 10,000.

In another version of expression cloning, a cDNA expression library, which mostly contains cDNAs that encode non-secreted proteins but also a few cDNAs that encode secreted proteins, is split into pools and transfected into cells suitable for expression of foreign proteins and peptides; typically COS cells are used. Conditioned media from the transfected COS cells are tested for the desired activity on a cell line. A pool of cDNAs that gave rise to the desired bioactivity is split into smaller pools and re-tested. The process is repeated until the candidate cDNAs are narrowed to one or a few.

For the chicken blastodermal cell culture, the media combinations to be tested are used on the cells as soon as the chicken blastodermal cells are plated.

The media is replaced with fresh media at least every other day. Ideally, to ensure that particularly labile factors are tested adequately, the media is replaced twice daily. Chicken blastodermal cells should be cultured for at least 3 days and ideally for 5 days or more before transfection with an Oct4-GFP vector. This is because under normal culture conditions, the percentage of cells in which the *Oct4* proximal promoter is active does not decrease significantly until after 3 to 4 days of culture. After that point the percentage drops off sharply. After 5 days of culture, the chicken blastodermal cells became crowded in the wells, which adversely affects the transfection and bioactivity efficiencies and may cause false negatives in the *Oct4* assay.

On day 2 or 3, a second set of chicken blastodermal cells are plated. These are used as a positive control to confirm that the Oct4-GFP vector is working.

On day 5, the chicken blastodermal cells are transfected along with the set of chicken blastodermal cells plated on day 2 or 3. A few wells are transfected with pCMV-GFP to confirm that the transfections are working. The rest of the wells are transfected with pOct4-GFP-PP.

The next day the wells are observed by fluorescent microscopy. The majority of pOct4-GFP-PP transfected-wells of the day 6 chicken blastodermal cells will have little or no GFP signal. The wells are carefully scanned for ones in which a significant level of green fluorescence is observed in either one or both duplicate plates. Wells that are GFP-positive indicate that the culture conditions unique to those wells are likely to maintain germline competence of cultured chicken blastodermal cells. The assay is repeated to confirm the identity of the culture conditions and media components.

As a final test for germline competence, the chicken blastodermal cells are cultured for various periods of time under the desired media conditions, harvested and used to form chick chimeras. The chimeras are then grown to maturity and tested for the ability to produce BR progeny, as described above. This test

provides confirmation of germline competence and the ability of the cultured chicken blastodermal cells to contribute to the germline.

If no factors are found to be positive using pOct4-GFP-PP, one repeats the screen using pOct4-GFP-DE or PE. Both the proximal and distal enhancers should be active in the presence of factors that promote germline competence. However some factors may promote a pluripotent state in chicken blastodermal cells which lacks the capacity to differentiate into germ tissue. The proximal or distal enhancer may be active in this state and lead to false positives. Alternatively, a germline-competent state could be achieved in which one or another enhancer is inactive.

All documents cited in the above specification are herein incorporated by reference. Various modifications and variations of the present invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in the art are intended to be within the scope of the following claims.

Table 1. EGFP expression driven by different promoters after transfection (% of surviving cells).

	Cell type			
	WEFs*		CBCs†	
plasmid	24 hrs after transfection	48 hrs after transfection	transfected after 1 day *in vitro*	transfected after 6 days *in vitro*
pOct4-EGFP-PE	0	0	5.5	0.2
pOct4-EGFP-PP	0	0	4.7	0.2
pOct4-EGFP-DE	0	0	2.8	0.1
pCMV-EGFP	30-40	40-50	30	25
pPGK-EGFP	5-10	10-20		
pEF1-alpha-EGFP	2-5	10-15		

*WEFs (see example 4 for description of WEFs) were transfected with DMRIE-C (see example 4 for transfection procedures) and the indicated plasmids. EGFP expression was determined by the percentage of surviving cells which express GFP 24 or 48 hours after transfection.

What is claimed is:

1. A method for identifying avian cells which can give rise to germline tissue, comprising:
   (a) introducing into avian cells an expression construct comprising (i) a marker gene; and (ii) control sequences sufficient for effecting expression of the marker gene in germline-competent cells, wherein at least one of the control sequences comprises a control sequence from a gene which is substantially expressed only in cells which are germline-competent and wherein the control sequences and the marker gene are operably linked; and
   (b) assaying for expression of the marker gene in the cells, wherein the expression of the marker gene in a particular cell indicates that said particular cell can give rise to germline tissue.

2. A method for identifying avian cells which can give rise to germline tissue, comprising:
   (a) introducing into avian cells an expression construct comprising an *Oct4* proximal promoter and a marker gene, wherein the *Oct4* promoter and the marker gene are operably linked; and
   (b) assaying for expression of the marker gene in the cells, wherein the expression of the marker gene in a particular cell indicates that said particular cell can give rise to germline tissue.

3. The method of claim 2, wherein said expression construct further comprises the distal enhancer of the *Oct4* gene, the proximal enhancer of the *Oct4* gene, or both.

4. The method of claim 2, wherein said expression construct comprises the promoter of the murine *Oct4* gene.

5. The method of claim 4, wherein said expression construct further comprises the distal enhancer of the mouse *Oct4* gene, the proximal enhancer of the mouse *Oct4* gene, or both.

6. The method of claim 5, wherein the construct comprises the proximal promoter and the proximal enhancer of the mouse *Oct4* gene.

7. The method of claim 6, wherein the expression construct comprises SEQ ID NO:1 operably linked to the marker gene.

8. The method of claim 2, wherein the avian cells are cultured or uncultured avian embryonic cells.

9. The method of claim 8, wherein the avian embryonic cells are chicken blastodermal cells from a chicken, turkey, duck, goose, or quail.

10. The method of claim 8, wherein the avian embryonic cells are selected from the group consisting of embryonic stem cells, embryonic germ cells, and primordial germ cells.

11. A method for identifying avian embryonic cells which can contribute to the germline of a recipient avian embryo, comprising:

(a) introducing into avian embryonic cells an expression construct comprising an *Oct4* proximal promoter; and a marker gene, wherein the *Oct4* promoter and the marker gene are operably linked; and

(b) assaying for expression of the marker gene in said cells, wherein the expression of the marker gene in a particular cell indicates that said particular cell can contribute to the germline of the recipient avian embryo.

12. A method of producing a germline-modified chimeric avian animal, comprising:

(a) introducing into avian embryonic cells an expression construct which comprises (i) a marker gene and an *Oct4* proximal promoter, wherein the marker gene and the *Oct4* enhancer, and the promoter are operably linked;

(b) identifying avian embryonic cells which express the marker gene sequence;

(c) transferring cells identified in step (b) to an avian embryo, wherein cells transferred to the avian embryo contribute to the germline tissue of the avian embryo; and

(d) allowing the avian embryo to develop to hatch.

13. A method of producing a germline-modified transgenic avian, comprising:

(a) introducing a transgene of interest into avian embryonic cells;

(b) before, after, or at the same time as step (a), introducing into the avian embryonic cells an expression construct comprising (i) a marker gene; and (ii) control sequences sufficient for effecting expression of the marker gene in germline-competent cells, wherein at least one of the control sequences comprises a control sequence from a gene which is substantially expressed only in germline-competent cells and wherein the control sequences and the marker gene are operably linked;

(c) identifying avian embryonic cells which comprise the transgene of interest and also express the marker gene of the expression construct;

(d) transferring cells of step (c) to an avian embryo, wherein the cells transferred to the avian embryo contribute to the germline tissue of the avian embryo; and

(e) allowing the avian embryo to develop to hatch,

wherein the germline tissue of the hatched avian embryo comprises the transgene of interest.

14. A method of producing a germline-modified transgenic avian animal, comprising:

(a) introducing a transgene of interest into avian embryonic cells;

(b) before, after, or at the same time as step (a), introducing into the avian embryonic cells an expression construct which comprises a marker gene and an *Oct4* proximal promoter, wherein the marker gene and the *Oct4* promoter, and the promoter are operably linked;

(c) identifying avian embryonic cells which comprise the transgene of interest and also express the marker gene of the construct;

(d) transferring cells of step (c) to an avian embryo, wherein at least some of the cells transferred to the avian embryo contribute to the germline tissue of the avian embryo; and

(e) allowing the avian embryo to develop to hatch,

wherein the germline tissue of the hatched avian embryo comprises the transgene of interest.

15. The method of claim 14, wherein said expression construct further comprises the distal enhancer of the *Oct4* gene, the proximal enhancer of the *Oct4* gene, or both.

16. The method of claim 14, wherein said expression construct comprises the proximal promoter of the murine *Oct4* gene.

17. The method of claim 16, wherein said expression construct comprises the distal enhancer of the mouse *Oct4* gene, the proximal enhancer of the mouse *Oct4* gene, or both.

18. The method of claim 17, wherein the construct comprises the proximal promoter and proximal enhancer of the mouse *Oct4* gene.

19. The method of claim 18, wherein the expression construct comprises SEQ ID NO:1 operably linked to the marker gene.

20. The method of claim 14, wherein said avian animal is a chicken, turkey, duck, goose, or quail.

21. The method of claim 20, wherein said avian embryo is a chicken embryo at a stage of development from about stage IV to about stage XII.

22. The method of claim 21, wherein the chicken embryo is at about stage X.

23. The method of claim 14, wherein the avian embryonic cells are cultured avian embryonic cells.

24. The method of claim 14, wherein the avian embryonic cells are cultured or uncultured chicken blastodermal cells.

25. The method of claim 14, wherein the avian embryonic cells are embryonic stem cells, embryonic germ cells, or primordial germ cells.

26. The method of claim 14, wherein the transgene of interest is integrated into the genome of the avian embryonic cells in step (a).

27. The method of claim 14, wherein in said step (a) the transgene of interest is introduced into the avian embryonic cells in a transgene addition vector, a gene-targeting vector, an artificial chromosome, or a retroviral vector.

28. A method of screening cell culture conditions for conditions in which the germline competence of the cells in the culture is maintained over a selected period of time, comprising:

(a) subjecting a population of cells to a set of selected cell culture conditions for said selected period of time;

(b) after said selected period of time has elapsed, introducing into the cells of the population an expression construct comprising (i) a marker gene; and (ii) control sequences sufficient for effecting expression of the marker gene in germline-competent cells, wherein at least one of the control sequences comprises a control sequence from a gene which is substantially expressed only in germline-competent cells and wherein the control sequences and the marker gene are operably linked; and

(c) assaying for expression of the marker gene in the population of cells, wherein the expression of the marker gene indicates that the selected culture conditions allow for the culture of germline-competent cells over said selected period of time.

29. A method of screening cell culture conditions for conditions in which the germline competence of the cells in the culture is maintained over a selected period of time, comprising:
    (a) subjecting a population of cells to a set of selected cell culture conditions for said selected period of time;
    (b) after said selected period of time has elapsed, introducing into the cells of the population an expression construct comprising an *Oct4* proximal promoter and a marker gene, wherein the *Oct4* promoter and the marker gene are operably linked; and
    (c) assaying for expression of the marker gene in the population of cells, wherein the expression of the marker gene indicates that the selected culture conditions allow for the culture of germline-competent cells over said selected period of time.

30. The method of claim 29, wherein said expression construct further comprises the distal enhancer of the *Oct4* gene, the proximal enhancer of the *Oct4* gene, or both.

31. The method of claim 29, wherein said expression construct comprises the promoter of the murine *Oct4* gene.

32. The method of claim 31, wherein said expression construct further comprises the distal enhancer of the mouse *Oct4* gene, the proximal enhancer of the mouse *Oct4* gene, or both.

33. The method of claim 32, wherein the construct comprises the proximal promoter and the proximal enhancer of the mouse *Oct4* gene.

34. The method of claim 33, wherein the expression construct comprises SEQ ID NO:1 operably linked to the marker gene.

35. The method of claim 29, wherein the cells are stem cells or germ cells.

36. The method of claim 29, wherein the cells are avian cells.

37. The method of claim 36, wherein the cells are avian embryonic cells.

38. The method of claim 37, wherein the cells are blastodermal cells from a chicken, turkey, duck, goose, or quail.

39. The method of claim 38, wherein said selected period of time is at least about four days.

40. The method of claim 29, wherein the cells are mammalian.

41. The method of claim 29, wherein steps (a)-(c) are applied to a plurality of population of cells in parallel, each of the populations of cells being subjected to a different set of culture conditions.

42. A method of selecting germline-competent avian cells from a population of avian cells, comprising:
   (a) introducing into a population of avian cells an expression construct comprising an *Oct4* proximal promoter and a selectable marker gene, wherein the promoter, and the selectable marker gene are operably linked; and

(b) subjecting the population of cells to conditions in which the expression of the selectable marker gene in an individual cell is required for survival of the individual cell.

43. The method of claim 42, wherein said avian cells are cultured blastodermal cells from a chicken, turkey, duck, goose, or quail.

44. An avian cell comprising an expression construct, wherein the expression construct comprises (i) a marker gene; and (ii) control sequences sufficient for effecting expression of the marker gene in germline-competent cells, wherein at least one of the control sequences comprises a control sequence from a gene which is substantially expressed only in germline-competent cells and wherein the control sequences and the marker gene are operably linked.

45. An avian cell culture comprising the cell of claim 44.

46. An avian cell comprising an expression construct, wherein the expression construct comprises an *Oct4* proximal promoter and a marker gene, wherein the *Oct4* promoter and the marker gene are operably linked.

47. The method of claim 46, wherein said expression construct further comprises the distal enhancer of the *Oct4* gene, the proximal enhancer of the *Oct4* gene, or both.

48. The method of claim 46, wherein said expression construct comprises the promoter of the murine *Oct4* gene.

49. The method of claim 48, wherein said expression construct further comprises the distal enhancer of the mouse *Oct4* gene, the proximal enhancer of the mouse *Oct4* gene, or both.

50. The method of claim 49, wherein the construct comprises the proximal promoter and proximal enhancer of the mouse *Oct4* gene.

51. The method of claim 50, wherein the expression construct comprises SEQ ID NO:1 operably linked to the marker gene.

52. The avian cell of claim 46 which is a blastodermal cell from a chicken, turkey, duck, goose, or quail.

53. The avian cell of claim 46 which is selected from the group consisting of an embryonic stem cell, an embryonic germ cell, and a primordial germ cell.

54. An avian cell culture comprising the cell of claim 46.

55. A germline-competent avian cell characterized by expression of a marker gene in said cell, wherein the expression of the marker gene indicates germline competence.

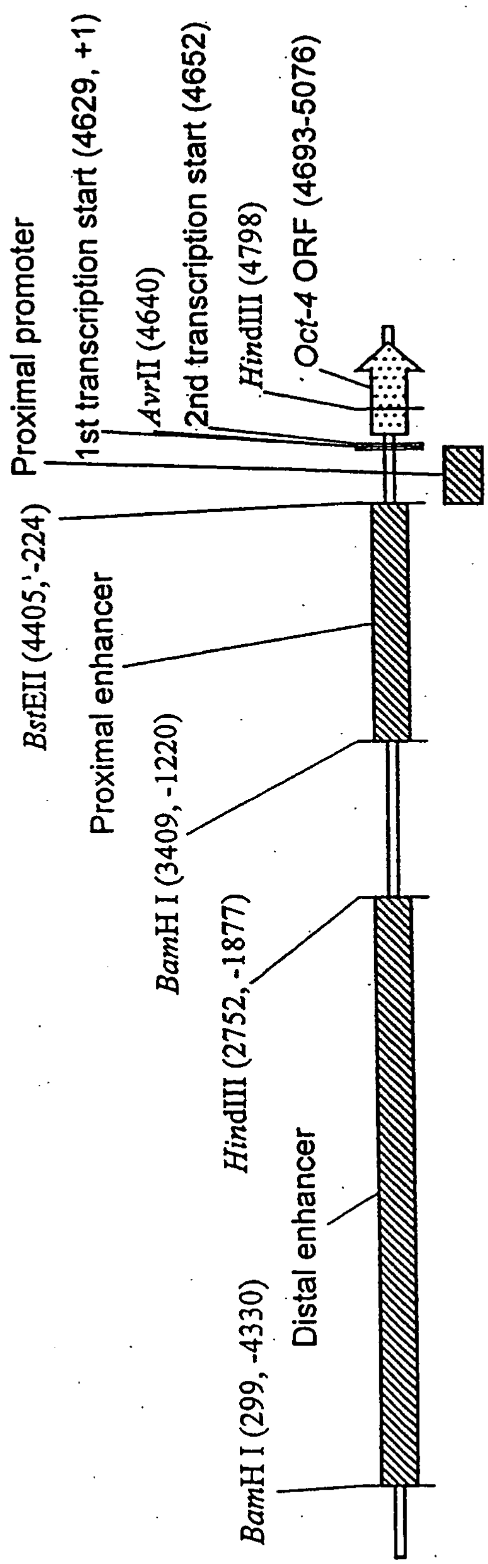
Proximal promoter
1st transcription start (4629, +1)
AvrII (4640)
2nd transcription start (4652)
HindIII (4798)
Oct-4 ORF (4693-5076)
BstEII (4405, -224)
Proximal enhancer
BamH I (3409, -1220)
HindIII (2752, -1877)
Distal enhancer
BamH I (299, -4330)

FIG. 1A

```
        HindIII
   1  AAGCTTTGTG AACTTGGCGG CTTCCAAGTC GCTGCCTTTA TTTAGGTCTT CCAACTAACT
  61  ATGGCACTGT TCCACAATGA ATGTATAGAA ATTGGGAGGT GAGCATGACA GAGTGGAGGA
 121  AACGGAAGAT TCATGGAGAG GGCCAGAGAG ATGGCCCCTC AGCCACCCTG GGGGATGACT
 181  TGGACCCATG TGGTAGAAGG AGGGGACTTT CACACATGTG CTATGTGTAG CTGTGTGTAG
 241  GTACATACAC ACCCTTAAAA TAAAACGCAA TTTTTTTTTC AAAGTCTCAG GGTGAATTTG
 301  GTGAAGTCGA TGAAGCTGAG GCAGGAGAAT TATCAGGAGT TCAAGGGCAG CTTGTTTTAT
 361  AGAGAAAGGT TCCATCTCTA CCTGATGAAG ACTACCATCA AGAGACACCC CCGCCCCCCA
 421  GGGCACCTAG AGCCACTGAC CCTAGCCAAC AGCTCAGGCG GGCTGGGCCC AGGCTCAGAA
 481  CTCTGTCCTG GCTATGTACA CTGTGGGGTG CTCTGGGCTT TTTGAGGCTG TGTGATTCAC
 541  CCTGGGGCCT TCGTTCAGGG CATGGTGTAG GAGCAGACAG ACAAACACCA TCCCTTGCAG
                                                                 BamHI
 601  ACAGGCACTC TGAGGGCTAT TCTCTTGCAA AGATAACTAA GCACCAGGCC AGTAATGGGA
         BamHI
 661  TCCTCAGACT GGGCCCAGAA AACCACTCTA GGAAAGTTCA GGGTAGGCTC TCTGCACCCC
 721  CTCCTCCTAA TCCCGTCTCC TTAGTGTCTT TCCGCCAGCA CAGGAATGGG GGAGGGGTGG
 781  GTGACGAGGA TGAACACCGG AGTCCCTGGA GGAAGGGAAG CAGGGTATCT CCATCTGAGG
 841  CTCTGTCTTT GAGGAGAGGT GGAGAGCTGG GGAAGTCTTG TGTGAGGGGA TTGGGGCTCA
 901  GGAGGGGGTT GGGGAGCAGG AAGTTGTCCC CAGGGGAGCC ATCCTGGCCC ATTCAAGGGT
 961  TGAGTACTTG TTTAGGGTTA GAGCTGCCCC TCTGGGGACC AGGATTGTCC AGCCAAGGCC
1021  ATTGTCCTGC CCCCTTCCCC CAGTCCCTCC CAGGCCCCTT TGAACCTGAA GTCAGATATT
1081  TCTTCTCTCT ACCCACCTCC CACCCGTTGG GTTTCTCCAC CCAGGAACTA GGCTGGAAGC
1141  TGGGATGAGG AGGTGGGGGA GGGAGAACTG AGAATCTTGA GGAAAGAGGC CCCGGCCTTA
1201  ACTGTGAGGG GATGGAGCCT GGGTGCAGGT CTTATGGGGG TTGGGGGTGG TTAGTGTCTA
1261  ATCTACCAAC CTGACAACAC AAGATGGATA CTGTGCTCTG AAAACGCAGA GCCAGCACTT
1321  CTCTGGGGTC TCTGGGGACA TATCTGGTTG GGGCTCGGGG TCCCATGGTG TAGAGCCTCT
1381  AAACTCTGGA GGACTGGAGG TGCAATGGCT GTCTTGTCCT GGCCTTGGAC ATGGGCTGAA
1441  ATACTGGGTT CACCCATATC TAGGACTCTA GACGGGTGGG TAAGCAAGAA CTGAGGAGTG
1501  GCCCCAGAAA TAATTGGCAC ACGAACATTC AATGGATGTT TTAGGCTCTC CAGAGGATGG
1561  CTGAGTGGGC TGTAAGGACA GGCCGAGAGG GTGCAGTGCC AACAGGCTTT GTGGTGCGAT
                                                BstEII
1621  GGGGCATCCG AGCAACTGGT TTGTGAGGTG TCCGGTGACC CAAGGCAGGG GTGAGAGGAC
1681  CTTGAAGGTT GAAAATGAAG GCCTTCCTGG GGTCCCGTCC TAAGGGTTGT CCTGTCCAGA
1741  CGTCCCCAAC CTCCGTCTGG AAGACACAGG CAGATAGCGC TCGCCTCAGT TTCTCCACCC
1801  CCACAGCTCT GCTCCTCCAC CCACCCAGGG GGCGGGGCCA GAGGTCAAGG CTAGAGGGTG
                                            AvrII
1861  GGATTGGGGA GGGAGAGGTG AAACCGTCCC TAGGTGAGCC GTCTTTCC
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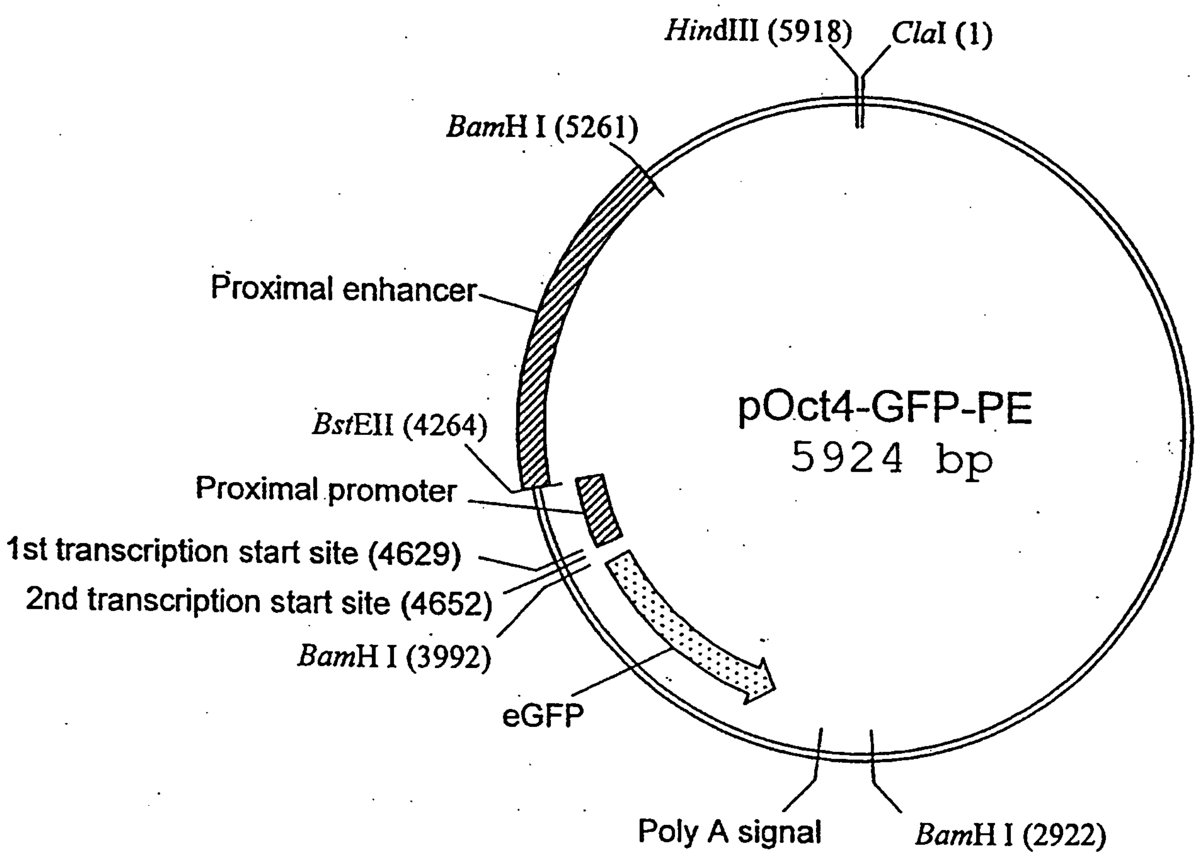
FIG. 1B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ClaI (1)
BamH I (5261)
Proximal enhancer
pOct4-GFP-PE
5924 bp
BstEII (4264)
Proximal promoter
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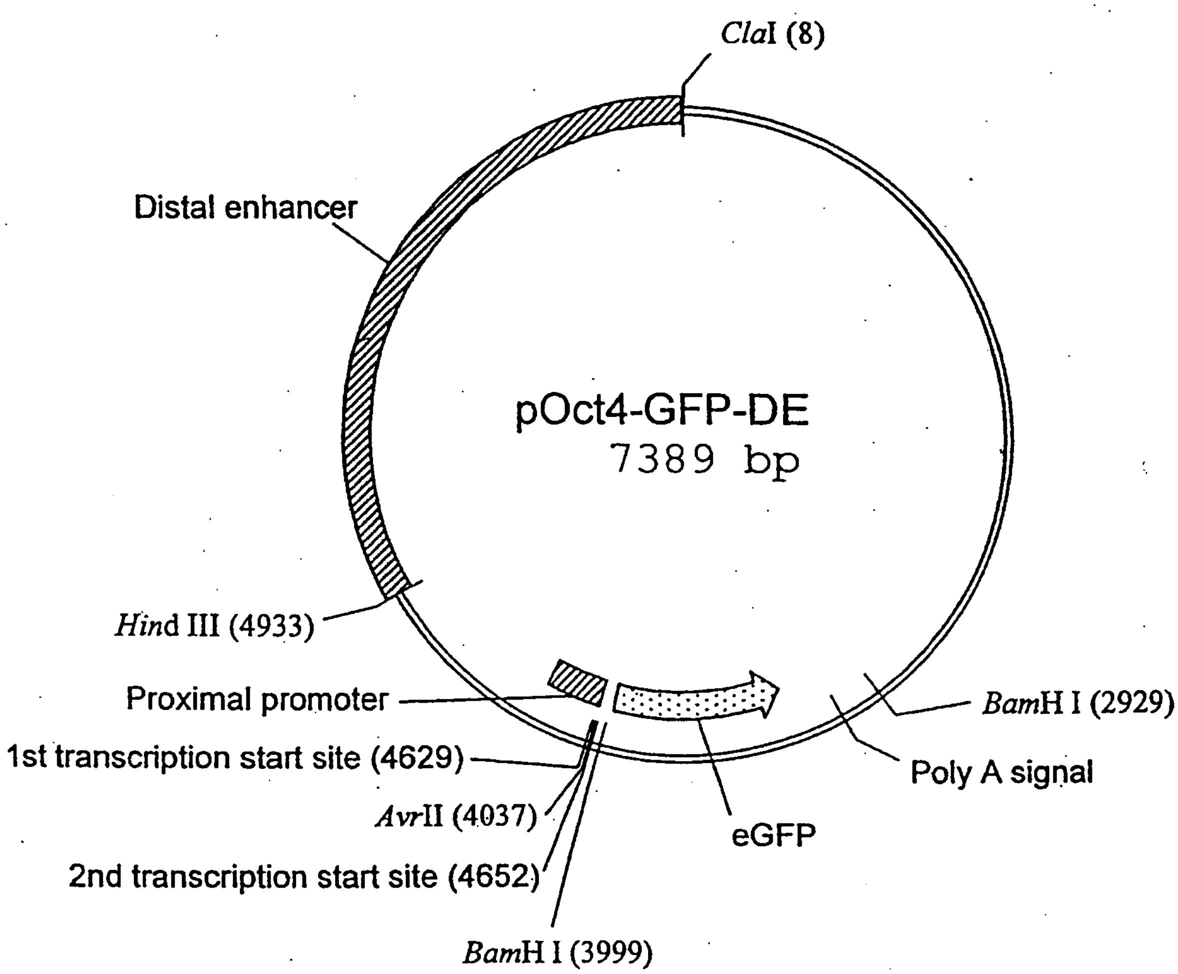
2nd transcription start site (4652)
BamH I (3992)
eGFP
Poly A signal
BamH I (2922)

FIG. 2A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7389 bp
Hind III (4933)
Proximal promoter
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AvrII (4037)
2nd transcription start site (4652)
BamH I (3999)
eGFP
BamH I (2929)
Poly A signal

FIG. 2B

BEST AVAILABLE COPY

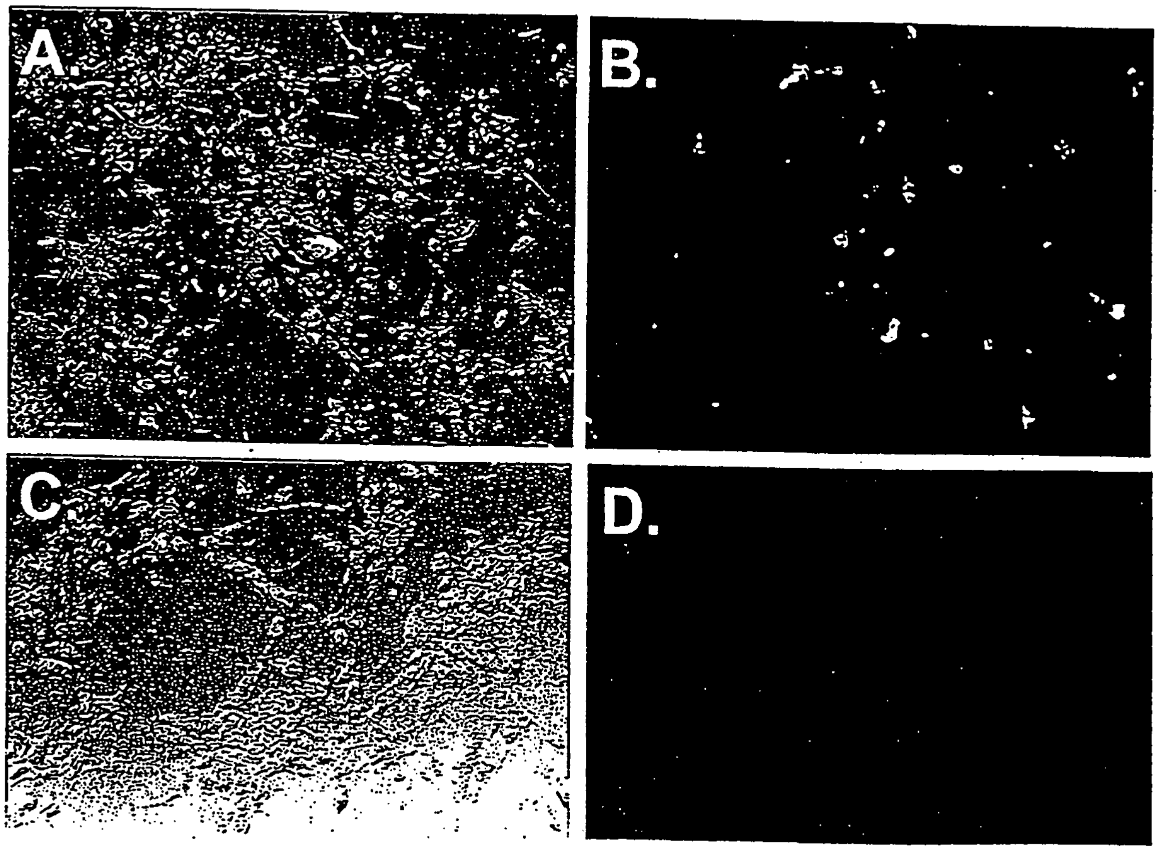
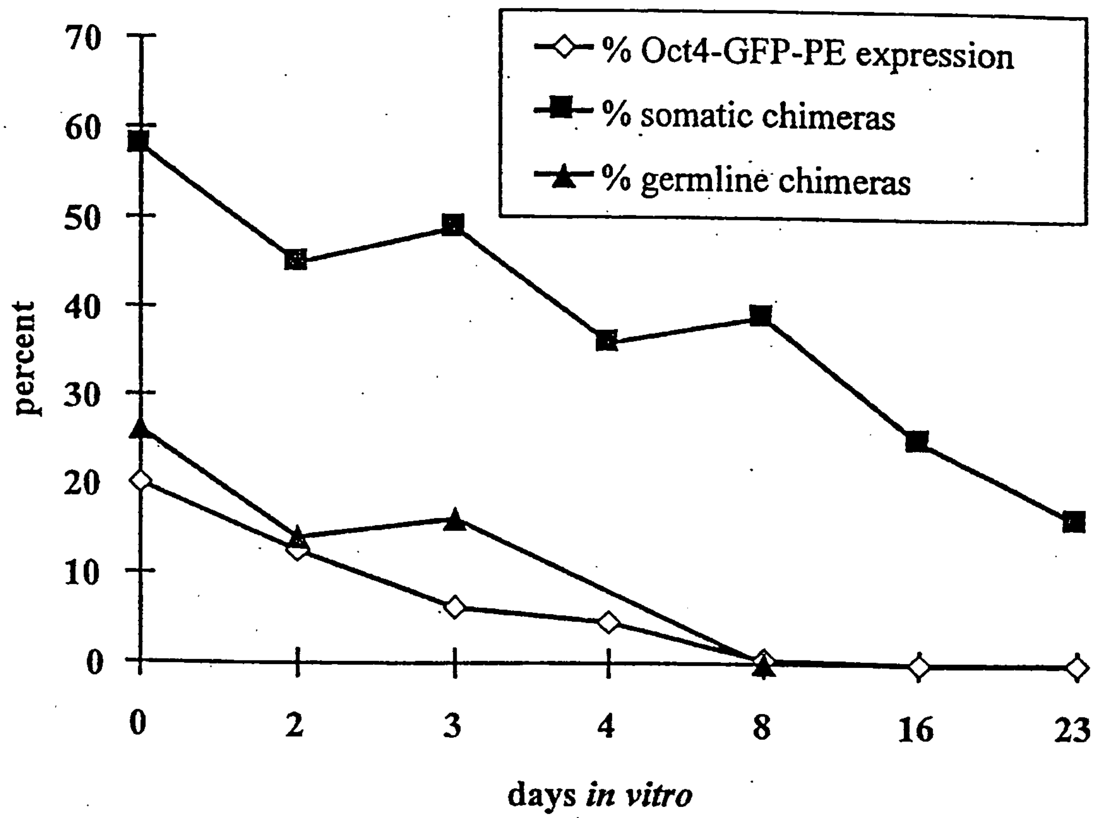


FIG. 3



% Oct4-GFP-PE expression
% somatic chimeras
% germline chimeras
70
60
50
40
30
20
10
0
percent
0
2
3
4
8
16
23
days *in vitro*

FIG. 4

Figure 5

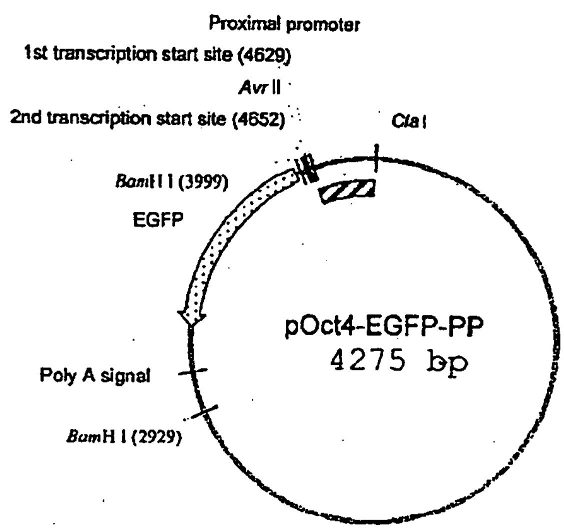

Proximal promoter
1st transcription start site (4629)
Avr II
2nd transcription start site (4652)
Cla I
BamH I (3999)
EGFP
pOct4-EGFP-PP
4275 bp
Poly A signal
BamH I (2929)

Figure 6

```
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 51  TCCTGGGGTC CCGTCCTAAG GGTTGTCCTG TCCAGACGTC CCCAACCTCC
     AGGACCCCAG GGCAGGATTC CCAACAGGAC AGGTCTGCAG GGGTTGGAGG

101  GTCTGGAAGA CACAGGCAGA TAGCGCTCGC CTCAGTTTCT CCACCCCCAC
     CAGACCTTCT GTGTCCGTCT ATCGCGAGCG GAGTCAAAGA GGTGGGGGTG

151  AGCTCTGCTC CTCCACCCAC CCAGGGGGCG GGGCCAGAGG TCAAGGCTAG
     TCGAGACGAG GAGGTGGGTG GGTCCCCCGC CCCGGTCTCC AGTTCCGATC
                                          AvrII
                                         -------
201  AGGGTGGGAT TGGGGAGGGA GAGGTGAAAC CGTCCCTAGG TGAGCCGTCT
     TCCCACCCTA ACCCCTCCCT CTCCACTTTG GCAGGGATCC ACTCGGCAGA

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SEQUENCE LISTING

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Speksnijder, Gord
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